Characterisation of two morphogenesis mutants in Arabidopsis thaliana

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By

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Abstract

In this thesis is described the characterisation of two morphogenesis mutants of *Arabidopsis thaliana*, with the aim of furthering our understanding of signalling in development. A thorough phenotypic analysis of both mutants is presented, with particular attention paid to the seedling root. The results of genetic analysis of *hydra2* are also presented.

The *hydra* mutants are characterised by a pleiotropic phenotype, with defective embryonic and seedling cell patterning, morphogenesis and root growth. The *HYDRA1* gene encodes a Δ8-Δ7 sterol isomerase, while *HYDRA2* encodes a sterol C14 reductase, previously identified as the *FACKEL* gene product. Seedlings mutant for each gene are similarly defective in the concentrations of the three major *Arabidopsis* sterols.

Reporter gene analysis showed mis-expression of the hormonally-regulated *DR5*, *IAA2* and *ACS1* promoters and of the epidermal cell file-specific *GL2* promoter in the mutants. The mutants also exhibit enhanced responses to auxin. The phenotypes can be partially rescued by inhibition of auxin and ethylene signalling, but not by exogenous brassinosteroids.

Evidence of abnormal activity of hormone-related membrane-bounds proteins and of altered membrane permeability to auxin is presented. A model in which correct sterol profiles are required for regulated auxin and ethylene signalling through effects on membrane function is proposed.

Declaration

The material contained in this thesis has not been submitted for a degree in this or any other University.

Statement of Copyright

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Stuart A. Casson, Paul M. Chilley, Jennifer F. Topping, I. Marta Evans, Martin A. Souter and Keith Lindsey (2002). The *POLARIS* Gene of *Arabidopsis* Encodes a Predicted Peptide Required for Correct Root Growth and Leaf Vascular Patterning. ***The Plant Cell*** 14, pp 1705-1721.

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Contents

List of Figures and Tables

Abbreviations

AA	Ascorbic acid
AAO	Ascorbic acid oxidase
ACC	1-aminocyclopropane-1-carboxylic acid
ADF	Actin Depolymerisation Factor
AGP	arabinogalactan protein
AVG	L-a-(2-aminoethoxyvinyl)-glycine
BA	6-benzylamino-purine
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
2,4-D	2,4-Dichlorophenolic acid
DIC	differential interference contrast (Nomarski)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease (an enzyme that breaks down DNA)
dNTPs	2'-deoxynucleoside 5'-triphosphates
EDTA	ethylene diamine tetraacetic acid, disodium salt
EGTA	ethylene glycol tetraacetic acid
EtBr	ethidium bromide
EtOH	ethanol
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
GUS	β-glucuronidase
gusA (uidA)	gene encoding β-glucuronidase
HAc	acetic acid
IAA	indole-3-acetic acid
IAN	indole-3-acetonitrile
IBA	indole-3-butyric acid
Ig	immunglobulin
JIM	John Innes monoclonal antibody
Kb	kilobase (length of 1000 nucleotides)
kg	kilogram
mRNA	messenger RNA
MS	Murashige and Skoog medium
MTSB	microtubule stabilisation buffer
NAA	α-naphthalene-acetic acid
NPA	1-N-naphthylphthalamic acid
NOA	1-Naphthoxyacetic acid
OxIAA	2-oxoindole-3-acetic acid
PAT	polar auxin transport
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PIPES	piperazine ethanesulfonic acid
RNA	ribonucleic acid
RNAi	RNA Interference
RNase	ribonuclease (an enzyme that breaks down RNA)
ROP	RHO-related GTPase from plants
rpm	revolutions per minute
SAM	shoot apical meristem

sdH_2O	sterile (autoclaved) distilled water
SE	standard error
SEM	scanning electron microscopy
SD	standard deviation
TAE	tris-acetate
T-DNA	transferred DNA
TIBA	tiiodobenzoic acid
Tris	tris(hydroxymethyl)-aminomethane
TRITC	
XET	Xyloglucan endotransglycosylase
X-Gluc	5-bromo-4-chloror-3-indoyl β-D-glucuronic acid
var.	variety
VLCFA	Very Long Chain Fatty Acid

Chapter 1

Introduction:

Signalling in Growth and Development

Introduction

Higher plants have been essential to the life of man, providing sustenance, medication and a source of important materials. Over time man has acted as a selection pressure upon the evolution of desirable traits in crop plants, unwittingly selecting genes that specify the phenotypic traits that were desired. Through an understanding of genetics, we are now able to select more precisely at the molecular level, and so an understanding of the genetic mechanisms that underpin plant growth and development is essential to our future manipulation of crops.

The growth and development of higher plants can be considered to be characterised by the execution of cell division, expansion and differentiation along two axes: the apical-basal axis and the radial axis. The radial axis is most clearly evident in dicotyledonous species as the concentric rings of cell layers in the seedling stem, hypocotyl and root, and an increase in size across this axis can arise from the generation of new cell layers following divisions in the vascular cambium in the older plant. The apical-basal axis can be defined by the patterning of functionally distinct structures, rather than cell layers, from the shoot apical meristem, to the hypocotyl and stem, to the root apical meristem.

Plant development can be considered to be plastic, as it must be flexible enough to allow the plant to adapt to the changing environment in which it finds itself. It achieves this through the integration of external signals with the co-ordination of growth that is mediated by hormones and other signalling molecules within the plant. Unlike humans who undergo a growth phase to adulthood and then cease growth, plants continue to grow throughout their lifetime, however the rate of growth is dependent upon its environment and the resources available in it. Through evolution species have become reproductively isolated and so followed their own adaptive path, becoming better equipped to live in certain environments and to mine the available resources. Consequently the plant kingdom exhibits a wide and varied range of structures that sometimes perform the same function, such as leaves.

Early taxonomists used physical characteristics to define species and to assign identity, but now we are learning more clearly, in this genomic age, that a number of the underlying genetic mechanisms are very similar (e.g. the genes that control floral patterning), and that some genes even overlap with humans in code and sometimes in

function (e.g. genes that regulate and control the cell cycle). We are also learning more about evolution as we compare genomes from different species and even from different kingdoms, and it is interesting to see how certain important genes have remained unchanged whilst others have diverged in form and function. However, to fully appreciate how different organisms with similar combinations of genes acquire different forms and life-styles, it is important to understand development. What makes these differences between species is thousands of years of evolution. Over time different patterns of gene expression have been established, so that despite the original genetic material being identical, different environments have created different usage of it.

Essentially then, development is the co-ordinated expression of genes and groups of genes in space and time. In plants the co-ordinators of this orchestra of genes are the signalling mechanisms that exist to interpret environmental cues, and the subsequent events that they bring about.

The aim of this first chapter is to introduce the major plant hormones, and then to show the importance of these signalling systems and their role in regulating development. We will firstly look at the model species *Arabidopsis thaliana*, and in particular focus on its use in a mutagenic approach to investigating development. Then we will look at the major hormones that regulate and control development, before seeing how these and other signal molecules orchestrate embryonic and post-embryonic development.

Part One: *Arabidopsis* and Mutagenesis

1.1 *Arabidopsis thaliana* as a Model Species

Arabidopsis thaliana is a small dicotyledonous plant of the Crucifereae family, and is more commonly known as thale cress. It is used as a model organism for plant genetic and developmental studies because it has many favourable characteristics for such studies, so much so in fact it has become known as the "Botanical *Drosophila*".

Arabidopsis' short life-cycle, which is complete in two months, it's small size and the fact that it has a high seed yield, make it suitable for cultivation on a large scale in a laboratory or greenhouse. It also has many advantageous genetic traits, such as having one of the smallest known plant genomes (0.2pg DNA), that consists of five haploid chromosomes. Also, in contrast to many other plant species, it does not have large amounts of repetitive DNA; roughly 80% of the genome is single-copy DNA (Meyerowitz, 1987). Because of the low amount of dispersed repetitive sequences, and the small size of the genome, chromosome walking and other gene isolation techniques are possible. These strategies are not often practical in other plant species, which require more time consuming approaches.

However, with the *Arabidopsis* genome now mapped a new set of approaches can be used to speed up the identification and manipulation of specific gene targets. There appear to be roughly 26,000 genes present in the genome, which seems to be a small number but it may become higher once we understand how important differential splicing is in plants. When classed together this gives *Arabidopsis* a proteome of between 11,000 to 15,000 members, which is similar to the functional diversity seen in *Drosophila* and *Caenorhabditis elegans*, however there are new protein families present as well as common ones missing. 69% of the genes present have homology to proteins of known function in other organisms, though only 9% have been characterised experimentally, and 30% are either plant specific or have no known homology to allow a functional role to be assigned. Common intracellular activities such as vesicle trafficking and the cell cycle are homologous across the kingdoms, whilst some divergent features include cytoskeleton organisation and cytokinesis, which have to take into account the presence of the plant cell wall. Interestingly, high numbers of genes have at least 5 other family members (The *Arabidopsis* Genome Initiative, 2000).

Genome Evolution

Analysis of the structure of the genome can reveal its evolutionary history. There appears to have been an entire genome duplication event, as well as subsequent gene loss and other more local gene duplication events. Polyploidy is a key factor in speciation and evolution, and it appears that there was a duplication event leading to the generation of a tetraploid ancestor roughly 112 million years ago. The problem with gene duplication means that genetic redundancy results, however the further back in time the duplication event, the greater the amount of functional divergence is possible. Such a restructuring of the genome could have been achieved by a number of large independent segmental duplication events rather than through tetraploid formation and stabilisation. However, when *Arabidopsis* is compared with other members of the Brassica family as well as with rice and tomato, a tetraploid ancestor is more confirmed (The *Arabidopsis* Genome Initiative, 2000).

Since the divergence from this polyploid, a number of other rearrangements have occurred, such as unequal crossing over, segmental inversions and gene translocations, giving the genome its present day form. Some gene redundancy exists, however the divergence of a large number of promoters and genes means that *Arabidopsis* is a useful tool because genetic mutagenesis can be carried out (The *Arabidopsis* Genome Initiative, 2000).

1.1.1 *Arabidopsis* is an Ideal System for Mutagenesis

The generation of genetic mutants is an approach that has been around for some time now, yet its effectiveness and power are only really being realised now that the *Arabidopsis* genome has been completed. By creating a mutant allele of a gene the influence of that gene on the phenotype can be assessed, and this can work in either a forward or a reverse genetic approach. A forward approach involves searching for mutants with specific kinds of phenotypic aberrations, such as looking for mutants in which the basal part of the embryo does not develop correctly. By using this approach one can find genes that may be of interest to the developmental process that is being investigated. However, because a phenotype may be created by a gene with an indirect relationship to the process in question, there is a risk that once the gene is cloned, it is difficult to understand how the mutant phenotype has been created.

In a reverse genetic approach, a gene of interest is found first, and then a mutation in that gene is sought from a population of mutants, or gene expression is ablated by antisense RNA or RNAi techniques. This approach is more direct but has only really been an easier and more advantageous approach since the genome was completed. With this approach the time from identification to an understanding of gene function is a lot faster, for reasons that will be discussed later.

Mutants can be generated through three broad methods, either by irradiation or chemical means, or by insertional mutagenesis.

Chemical mutagenesis is the major method used to create novel mutants, as it is quick and can generate a wide spectrum of stable genetic lesions (Topping and Lindsey, 1995). Ethyl methane sulphonate (EMS) is one of the most commonly used mutagenic agents. EMS reacts with guanine, where it causes an ethyl group to be added to the number 7 nitrogen. Alkylation of the guanine allows it to pair with thymine so that during replication the complementary strand receives thymine rather than cytosine. This is known as a base substitution of the transition type (King and Stansfield, 1997). The generation of point mutations is highly advantageous, as it allows multiple alleles of a gene affecting a given developmental process to be identified. This is the strength of this method, and it is often found that point mutations accumulate within the regions of the gene that are crucial to function. The severity of the phenotype can then be compared between alleles with mutations in different regions of the gene, giving more insight into the areas of the gene/protein that are crucial for activity or interaction with the substrate or other proteins/regulatory components (Topping and Lindsey, 1995).

The ultimate success of a forward genetic approach however, is the quality of the screen used to look for mutants. Mutants are selected on specific criteria, depending on what type of mutants are required, such as selecting for auxin insensitive mutants on medium containing auxin. It is, however, difficult to screen for some types of mutations as they may be homozygous lethal, or may be masked by gene redundancy. Also if a more lethal mutation has been produced elsewhere in the genome, then the plant will not be viable. This highlights the less predictable, more uncontrollable, side of the approach when it is compared to using other methods.

Another potential disadvantage with EMS mutants generated in a forward approach is that they require more time consuming approaches to cloning the disrupted gene. Methods have improved in recent years, such as CAPS marker analysis, which can pinpoint an area of a chromosome (Baumbusch *et al.*, 2001), and then chromosomal walking can be done. Alternatively predicted genes within an area of a chromosome predicted by CAPS mapping can be found on the genome database and then sequentially cloned looking for the mutation.

Insertional mutagenesis involves the integration of a DNA fragment (T-DNA) ectopically into the plant genome, a technique also known as gene tagging, which often causes a total loss of function in the disrupted gene (Topping and Lindsey, 1995). Transformants can be selected on antibiotic selection plates, and then subjected to the screening process.

T-DNA mutants have the advantage of being able to use the T-DNA as a tag to clone the flanking sequences around it, and therefore the gene, such as by plasmid rescue (Behringer and Medford, 1992). T-DNA generated mutant populations are therefore very popular when looking for a mutation within a certain gene, as in a reverse genetic approach. Groups who have established such collections either sequentially clone flanking regions of each T-DNA and then put the information onto a BLAST database for searches to be conducted over the Internet, such as at the Salk Institute (at signal.salk.edu/tabout.html) or through the *Arabidopsis thaliana* Insertion Database run by the John Innes Centre. Alternatively pooled DNA is supplied so that PCR can be conducted by the researcher (e.g. the Maddison lines or TAIR at arabidopsis.org) or the researcher sends off their PCR primers for the organisation to screen for them (e.g. the Syngenta lines). The result of this work is that a large number of independent T-DNA collections are now available to the research community over the Internet, facilitating the rapid recovery of interesting mutants in a more direct way.

This can be particularly advantageous when looking for lethal mutations, such as the isolation of the *auxin binding protein1* mutant in *Arabidopsis* (Chen *et al.*, 2001). Initially the search for the mutant proved difficult, the gene is so important that the embryos in the knock-out plants arrest development at a very early stage. By conventional forward genetics this mutant would not have been recovered unless a specific embryogenesis screen was being used. However at the time auxin's role in

embryogenesis was not fully understood, and so it took until just recently when T-DNA tagged mutant populations became more widely available that the mutant was isolated. The line can only be worked on in the heterozygous state, another complication that the forward approach does not accommodate very well unless the screen is appropriate, as embryo lethal mutations will not be seen in a screen for seedling defects.

When insertional mutagenesis was first used, one major disadvantage was that it was often very difficult to identify the mutants that had resulted from the T-DNA insertion against the background of somaclonal variation that naturally arose during the regeneration process, i.e., tissue culture. However new transformation techniques have been developed that do not require such regeneration, and therefore do not have the same risk of generating somaclonal variation. Dipping has now become the popular method of transformation, and it is a system that *Arabidopsis* lends itself to very well (Clough and Bent, 1998). Plants are dipped into a solution of *Agrobacterium* harbouring the construct of interest and then left for a few days. The *Agrobacterium* infect the egg cells within the immature flowers prior to self-fertilisation. T1 seeds are then collected and germinated on antibiotic selection media, with the resistant seedlings being grown onto the next generation. The only major disadvantage with all mutagenesis techniques is the time that sometimes needs to be taken to reduce the number of mutations within each line by backcrossing. In the case of T-DNA insertion this means reducing the number of T-DNAs within the line, although sometimes multiple T-DNA insertion events can occur at the same locus, making plasmid rescue either difficult or impossible.

Double, Triple and Quadruple Mutants

If a gene family is being investigated then the generation of multiple mutants can be achieved through crossing. Similarly two or three mutants within a pathway may be crossed to determine where in the pathway they act, or what the cumulative effect on the phenotype is when all are knocked out. Redundant genes can be seen in this case, but identification of a redundant gene family through conventional forward genetic techniques mentioned above is very rare because without a phenotype to select on, the mutations will not be selected for further analysis and discarded at an early stage.

Trapping Promoters of Interesting and Redundant Genes

One way of overcoming genetic redundancy is to use a promoter-trap. A promoter-trap uses promoter-less reporter genes, such as the *uidA* (*gusA*) gene encoding β-glucuronidase (GUS) gene (Topping and Lindsey, 1995; Jefferson *et al.*, 1987). Because the construct is promoter-less, the natural promoter of the gene it inserts into will drive its expression, so that GUS is only seen in cells in which the disrupted gene is normally expressed. The expression pattern can be used to help identify genes and promoters of interest, such as genes expressed only in the root, or genes that are activated upon pest or pathogen attack. Also, the insertion of the T-DNA carrying the promoter-less construct may cause a knock-out akin to that of the normal T-DNA insertion event mentioned above. This approach therefore offers two advantages, an immediate idea of the spatial and temporal expression of the gene, and if it is not functionally redundant, a mutant phenotype (Topping and Lindsey, 1995). Also, homozygous lethal phenotypes can be studied in the heterozygous state because the disrupted gene's role can be inferred from the expression patterns seen in the heterozygous plants. Promoter trapping even allows mutations in polyploid species to be isolated and studied.

1.1.2 Conclusions

Arabidopsis is an excellent model system in which to study a number of aspects of development, and to understand the molecular mechanisms that underpin these processes. The application of genomic and proteomic technologies will only quicken the search for interesting genes and an understanding of their function and mode of action. The next section will introduce the major plant growth regulators or plant hormones, and show how the advent of molecular biological approaches has transformed our understanding of the different hormones; how and where they are made, how they are transported, and how they bring about changes in gene expression.

Part Two: Phytohormones

Introduction

The best characterised group of endogenous signals are naturally occurring organic substances with the ability to influence plant physiological processes at low concentrations. They are referred to as plant hormones or phytohormones. The whole concept of phytohormones was derived from the mammalian field, where it involves a localised place of synthesis in a specialised tissue, an endocrine gland, followed by secretion into and transport through the blood stream to its specific target tissue. This is not the rule for plant hormones. They can be synthesised by a wide range of tissues, with some being transported but the majority acting at their site of synthesis. Therefore the mammalian paradigm must be recognised as the wrong analogy, and replaced with the more specific plant one. Plant hormones are therefore often referred to as plant growth regulators rather than as hormones, but this term actually encompasses all compounds, both natural and synthetic, that can evoke a physiological response.

Plant hormones have been known about for some time now; Darwin's experiments on phototropism in coleoptiles indicated the existence of transported signals which he described in his book 'Power of Movement in Plants' in 1880. An influential publication in the field came in 1937 when Went and Thiemann published their book entitled "Phytohormones" (Went and Thiemann, 1937). However it was not until the late 1970s and early 1980s when the mutagenesis approaches mentioned in the previous section were beginning to be widely used, and it was from the manipulation of this powerful technique in *Arabidopsis* that great strides were made in our understanding of how plant hormones function within development. Genetic approaches have allowed the researcher to interrupt signalling pathways within the plant without the concerns of external chemical application. The genomic age has only served to increase the speed at which groups can identify and investigate aspects of hormone biology, and the last few years have seen an explosion in all areas.

Plants hormones fit into the developmental process by allowing the plant to make the many "yes and no" decisions it needs to make, in order for it to be able to respond in the most appropriate manner to the environment in which it finds itself. Plants are capable of both determinate and plastic growth, which they are able to combine

through their meristems to give the level of environmental responsiveness that they need in order to survive. Plant hormones are the biological molecules that direct these responses. Indeed, nearly all physiological responses of a plant can be attributed either directly to a single hormone or to the interactions of different hormones. Temporal and spatial changes in phytohormone concentrations are believed to regulate many aspects of plant development (King *et al.*, 1995).

The five classical classes of phytohormone are auxins, ethylene, cytokinins, gibberellins and abscisic acid (ABA). Many researchers have recently argued that brassinosteroids deserve recognition as the sixth class of plant hormone; brassinosteroid auxotrophic and insensitive mutants indicate that brassinosteroids are essential for proper plant development and play an important role in the control of cell elongation (Kauschmann *et al.*, 1996; Clouse, 1996). Jasmonates, polyamines, salicylic acid, lipooligosaccharides and peptides have also been discussed as plant hormones (Lindsey *et al.*, 2002; Palme *et al.*, 1996; Davies, 1995).

It is important to understand the synthesis, transport and action of the major phytohormone classes in order to understand many of the developmental patterning events that we will look at in the next two sections. However, in the interests of space we will restrict our analysis here to auxin, ethylene, cytokinin, and brassinosteroids.

1.2.1 Auxin

Auxin is one of the most important phytohormones, and has been known about longer than any other; Darwin, although unable to identify it chemically, was the first to demonstrate phenotypically that a diffusable agent was important for tropic growth. In 1928 Went succeeded in elucidating this signal compound and termed it auxin, describing it as an "Organic substance which at low concentrations promotes growth (cell enlargement) along the longitudinal axis of the shoot, and inhibits the elongation of roots" (Went, 1928). Auxin is therefore the oldest and physiologically best characterised plant hormone.

Auxin research has exploded in the last six years, and the number of scientists working on aspects of auxin biology has created a highly competitive and exciting area.

Consequently a number of papers have been published recently, and there are still a number of interesting papers awaiting publication. Where possible published material has been used, and where work remains unpublished, personal communication has been cited.

1.2.1.1 Auxin Metabolism: Biosynthesis, Conjugation, and Breakdown

There are a number of naturally occurring forms of auxin, such as indole-3-butyric acid (IBA), 4-chlorindole-3-acetic acid (4-Cl-IAA), and phenyl acetic acid, however little is known about their physiological role. There are also a number of synthetic auxins with higher metabolic stability, such as 1-napthyl acetic acid (1-NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D), which are commonly used in commercial applications. The majority of work however has focused on the major endogenous auxin, indole-3-acetic acid (IAA).

Plants have evolved elaborate systems for regulating cellular levels of IAA, and it is now clear that they synthesise, inactivate and catabolize IAA by multiple pathways, and multiple genes encode multiple enzymes within a pathway. This has made the elucidation of IAA metabolism and the isolation of mutants very difficult indeed, as there are a number of pathways to make and inactivate auxin within the plant (Normanly and Bartel, 1999).

IAA can exist as free IAA, believed to be the bioactive form (Taiz and Zeiger, 1991), or as inactivated IAA, reversibly through conjugation, or irreversibly by oxidising IAA or IAA-conjugates (Normanly and Bartel, 1999). Conjugation can be through ester linkages to sugars and myoinositol, or to amino acids and small peptides through amide linkages (Cohen and Bandurski, 1982). Free IAA concentrations are co-ordinately regulated through the interplay of biosynthesis, conjugate formation, conjugate hydrolysis to free IAA, irreversible oxidation, and transport. This co-ordinated control is a very dynamic process that responds to developmental and environmental signals (Normanly and Bartel, 1999).

A number of possible pathways for IAA biosynthesis have been proposed, but for many years it was assumed that tryptophan (Trp) was the sole precursor of IAA. Now it is clear that both Trp-dependent and Trp-independent pathways exist (Wright *et al.*

1991; Normanly, Cohen and Fink 1993). It appears that Trp-dependent pathways predominate during early embryogenesis and seed germination, whilst Trp-independent predominate during later stages of embryogenesis and during vegetative growth (Normanly 1997).

Normanly (1993) postulated that the major pathway for auxin biosynthesis incorporates indole or indole-glycerol phosphate, through indole-3-acetonitrile (IAN) to IAA. A confirmation of this came with the isolation of nitrilase enzymes which convert IAN to IAA; three *Arabidopsis NITRILASE* genes have been cloned (*NIT1, NIT2* and *NIT3*) (Bartel and Fink, 1994; Bartling *et al.*, 1994; Normanly *et al.*, 1997). However, the current state of play is still no clearer, and the possibility still remains that one or more of the proposed pathways may have no physiological relevance.

Evidence is now also emerging of the developmental and spatial regulation of conjugated forms of auxin with *Arabidopsis*; 2-oxoindole-3-acetic acid (OxIAA) is the most abundant form in all tissues, while IAA-Glu and IAA-Ala show high levels in the leaves, with IAA-Leu showing higher levels in the roots rather than the shoots. Also, IAA-Asp now appears to be an irreversible catabolite form rather than one for storage. Together these results suggests that different conjugates play different roles in IAA metabolism. Also, high levels of free IAA are matched by high levels of conjugates, indicating rapid turnover (Kowalczyk and Sandberg, 2001).

Auxin metabolism therefore gives the plant a number of ways in which to control the levels of auxin made and the levels of active auxin available through conjugation and re-activation. But in order to understand this within a developmental context, we need to know where auxin is being made and how it is being transported within the plant, in space and time.

Where is Auxin Made?

The major sources of auxin in the plant were believed to be the growing regions such as the shoot apex and young leaves (Normanly *et al.*, 1997), and this has now been demonstrated through the use of highly sensitive liquid chromotography-electrospray tandem mass-spectroscopy. Göran Sandberg's group in Sweden has led the way in establishing a system in which to measure small amounts of active and conjugated forms of auxin within very small amounts of plant tissue (50-100mg of tissue)

(Kowalczyk and Sandberg, 2001). Through studying the development of *Arabidopsis* with respect to levels of auxin within organs, an understanding of the role of auxin, as well as its place of synthesis and the possible destination of its transport is being elaborated. The technique that Sandberg's group have refined is more accurate than the total auxin measurement method that has been used for a number of years, which can generate a number of artefacts because conjugated forms are hydrolysed to active forms in the extraction process (Kowalczyk and Sandberg, 2001).

After germination the cotyledons are the major source of auxin as conjugated forms are activated and then transported towards the roots. At ca. 5-6 days post germination the first leaves start to develop, at which point there is an increase in the levels of free and conjugated forms of auxin, with the highest levels of free auxin associated with areas of high cell division (Kowalczyk and Sandberg 2001). This level decreases as the cells in the leaf switch from division to expansion, and when expansion finally stops the level decreases further. Concomitant with this increase in auxin levels in the developing leaves, on ca. day 5-7 there is a pulse of auxin released from the young organs, which reaches the root tip by ca. day 7 or 8 (Bhalerao *et al.*, 2002). This pulse of auxin from the first leaves is a light dependent process, and is only produced by the first true leaves (Bhalerao *et al.*, 2002). The pulse of auxin travelling down the plant accumulates at the hypocotyl-root junction, and drives lateral root development, but does not drive lateral root initiation (Casimiro *et al.*, 2001; Bhalerao *et al.*, 2002).

A gradient of auxin is established in the root tip, with the highest levels in the most apical 1mm, at around days 3-6 post germination. The root tip is thus established as a source of auxin before the pulse reaches the root tip, which creates a steeper gradient and which leads to an increased capacity for auxin biosynthesis in the root by day 10 (Bhalerao *et al.*, 2002). The pulse is not generated in the dark, and neither is the root tip gradient. Together these results show us that the development of the root system is dependent on the establishment of photosynthesis in the shoot, which leads to the first leaves generating the pulse of auxin that leads to the elaboration of the roots to supply the shoot with the water and nutrients it requires.

1.2.1.2 Auxin Transport

Auxin is the only plant hormone to be transported in polar manner throughout the plant, a process that is clearly important as many developmental processes can be disrupted by altering this transport system. Auxin transport has therefore been an important research area because of the number of developmental processes that require auxin transport, and because of its fundamental importance in understanding auxin physiology as well as metabolism. There are two transport main transport systems, the fast non-directed phloem auxin transport and the slower, directed, so-called polar auxin transport.

Non-Polar Auxin Transport Through the Phloem

The evidence for auxin transport through the phloem comes from experiments with radioactively labelled auxin (Morris and Thomas, 1978). The labelled auxin was applied to leaves and was later detected in the phloem elements of the leaves and in the vascular bundles of the stem. Transport occurred in both basipetal and acropetal directions of the shoot as well as of the root (Lomax *et al.*, 1995). This non-polar auxin transport system is coupled with the transport of assimilates (e.g. sugars), runs relatively quickly (5-20cm/h) and also transports the inactive auxin conjugates (Lomax *et al.*, 1995). Further experiments with radioactively labelled auxin in pea established the connection between the non-polar and the polar transport streams. The labelled auxin transported within the phloem (non-polar) was later detected in the polar transport system (Cambridge and Morris, 1996).

The Polar Auxin Transport System

Morris and Thomas (1978) also showed that radioactively labelled auxin applied to stems was found to concentrate in the cambium and adjacent partially differentiated xylem elements. Previous reports had implicated the parenchyma next to the phloem as the site of polar auxin transport (Goldsmith, 1977). Polar auxin transport requires energy, is specific for active free auxins and is considered as the main auxin transport system (Lomax *et al.*, 1995). Polar auxin transport in the stem runs basipetally at a velocity of 5-20mm/h towards the root, and there acropetally to the root tip. In the root tip another stream exists, taking the auxin delivered acropetally to the root tip and moving it basipetally through the root epidermis (Lomax *et al.*, 1995).

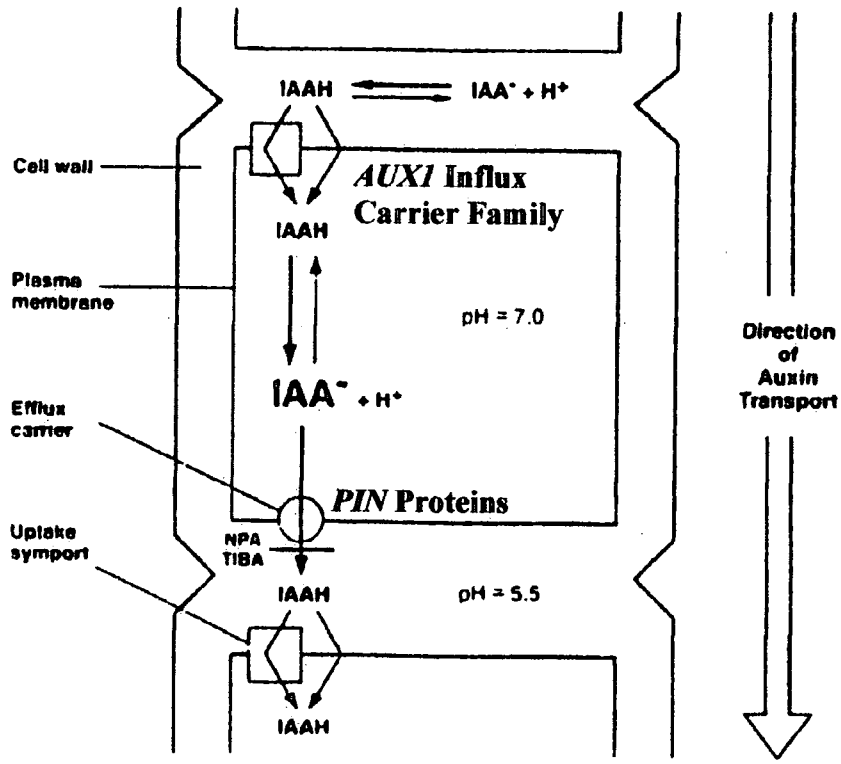
A major research question therefore was how this polar system was organised and regulated. Experiments with protein synthesis inhibitors suggested the existence of

specific transport proteins, and transport studies demonstrated cell to cell transport. All these experimental efforts culminated in the formulation of the chemiosmotic theory (Rubery and Sheldrake 1974; Raven 1975).

The Chemiosmotic Theory

The theory describes a mechanism for apoplast-symplast movement and postulated the existence of specific auxin efflux carrier proteins. IAA in the cell wall space enters the cell by diffusion. In the more acidic environment of the cell wall space (pH 5.5) IAA exists mostly in a protonated form (IAAH), and it is this non-charged lipophilic form that can diffuse easily through the plasma membrane. The more basic cytoplasm (pH 7.0) causes the IAAH to dissociate and the resulting IAA^- anion is then 'trapped' in the cell due to its low membrane permeability. IAA^- can exit the cell only via an anion efflux carrier. Metabolically-maintained pH differences across the plasma membrane provide the driving force for transport, whilst the basal location of the efflux carrier provides its polar nature (Rubery and Sheldrake 1974; Raven 1975).

Figure 1.2.1.1 Model of the Chemiosmotic Theory



(Figure taken from Davies *et al.*, 1995).

Later the existence of an auxin-specific influx carrier was also postulated (Goldsmith, 1977), specifically working as $IAA^-/2H^+$ co-transporter, an anion symport carrier (Benning, 1986; Lomax *et al.*, 1995).

The Influx Carrier: The *AUX1* Gene Family

A disruption in the *AUX1* gene provoked auxin and ethylene resistance and also affected root gravitropism in *Arabidopsis* seedlings (Bennett *et al.*, 1996). The corresponding gene encoded a 485 amino acid protein containing 11 predicted transmembrane domains, and sharing similarity with plant amino acid permeases, suggesting that this protein might be involved in the uptake of the tryptophan-like IAA. Recently the *AUX1* gene product has been shown indirectly to be the auxin influx carrier; *aux1* mutants are resistant to 2,4-D, a synthetic auxin analogue that requires the influx carrier to enter the cell, but are susceptible to NAA, a synthetic auxin analogue that can move freely through the membrane without the need for the influx carrier (Marchant *et al.*, 1999). Wild type plants can be induced to phenocopy the *aux1* mutant by supplying 1-naphthoxyacetic acid (1-NOA), a chemical that specifically inhibits the influx carrier (Parry *et al.*, 2001).

Promoter-GUS and immunolocalisation of the protein show that it is expressed specifically in the vascular initials, distal root cap cells and the lateral root cap, localised to opposite side of the cell to the efflux carrier. The localisation of it to the vascular initials suggests a role in unloading auxin from *PIN1* through the vascular tissues. The columella is important for sensing gravity and auxin is preferentially redistributed towards one side of the root through the lateral root cap and back in to the cortex and epidermal cells where the response is mediated. *IAA2::GUS* is also reduced in the regions behind the root tip, in the cortex and epidermis, suggesting that no auxin is being fed back out from the root tip (Swarup *et al.*, 2002).

A reverse genetic search through the *Arabidopsis* genome has shown that there is a small family of four *AUX* genes. The other three members have been designated *LIKE AUX1* (*LAX*) genes. While the family exhibit a high degree of amino acid sequence conservation (73-82% homology), the number and position of each of the genes introns indicates the evolutionary relationship between the four family members. Assuming that the addition of introns is a measure of evolving genome complexity, *LAX2* would

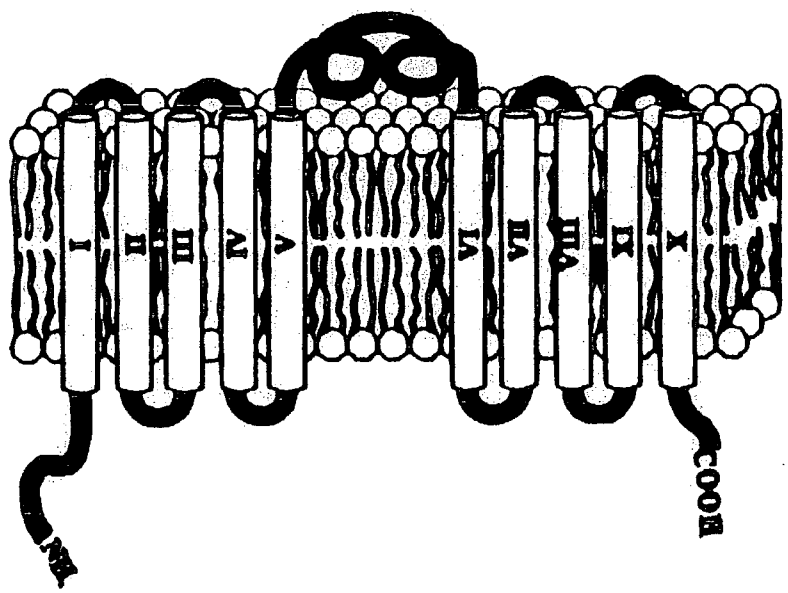
represent the founding member of the family, whilst *AUX1*, *LAX1* and *LAX3* have evolved later through gene/genome duplications (Parry *et al.*, 2002).

The four members show some overlapping expression within the root, as well as expression in the shoot. In particular they seem to be associated with vascular tissues and in phloem loading of auxin. This initial data suggest exciting things are ahead, and with the isolation of T-DNA mutants in all three *LAX* genes now available, double and triple and quadruple mutants will only help to show how important these genes are in different processes (Swarup *et al.*, 2002).

The Efflux Carrier: The *PIN* Gene Family

The family of genes shown indirectly to be the efflux carriers, has homology to bacterial transporters. They are known as *PIN* proteins because of the phenotype of the first mutant isolated, *pin-formed1*. In this mutant the inflorescence lacks any lateral organs, and instead just forms a pin-like tip that can be phenocopied if plants are supplied with the phytotropin NPA (Okada *et al.*, 1991). *PIN1* was cloned by transposon tagging and found to encode a 622 amino acid protein with 10 putative transmembrane domains (Galweiler *et al.*, 1998). The diagram below shows the predicted topology of the protein.

Figure 1.2.1.2 Topology of the *PIN* Family Proteins



(Figure taken from Palme and Galweiler, 1999).

From the identification of this sequence the rest of the family were found using a reverse genetics approach. To date nine *PIN* genes have been identified whilst more than twelve different *PIN* homologues have been found in *Arabidopsis*. *PIN* genes have also been identified in maize, rice and poplar, with high conservation between monocot and dicot species indicating a conserved function for PIN proteins throughout the plant kingdom (K. Palme, personal communication).

Using antibodies raised against a portion of the protein of each family member, and by confirming expression pattern with *in situ* hybridisation and immunogold labelling, the developmental expression patterns of each member has been worked out. Members of this family of transporters have different expression patterns within time and space, and are developmentally regulated. This offers the plant a means by which auxin can be transported precisely. The key therefore to understanding the direction of auxin movement within a tissue, and therefore the response of the plant, is the cellular localisation of these efflux carrier proteins. *PIN1* was localised at the basal end of elongated parenchymatous xylem and cambial cells in *Arabidopsis* inflorescences (Galweiler *et al.*, 1998), as well as on the acropetal side of stele cells in the root (J. Friml, unpublished results, personal communication). In the leaf it is also associated with the vascular tissue, which follows Sach's canalisation hypothesis (Sachs, 1991).

PIN2 is localised to the basal end of the cortex and epidermal cells in the root (i.e. transporting back up the root), as well as on the periclinal side of the cortex cells and the basal end of lateral root cap cells (Muller *et al.*, 1998; J. Friml, unpublished results, personal communication). The *pin2* mutant is agravitropic, and was later found to be allelic with *agravitropic1 (agr1)*, *wavy root6* (*wav6*) and the *ethylene insensitive root1 (eir1)* mutants (Luschnig *et al.*, 1998; Chen *et al.*, 1998; Utsuno *et al.*, 1998). *agr1* mutants show altered root gravitropism, increased root-growth sensitivity to auxin, and decreased sensitivity to ethylene (Chen *et al.*, 1998). *eir1* mutants are also agravitropic as well as having a reduced sensitivity to ethylene. GUS reporter expression is increased in wild-type plants by the presence of ethylene, which indicates that these cells may have increased auxin levels (Luschnig *et al.*, 1998).

PIN3 is localised in the first row of differentiated columella root cap cells, immediately underlying the columella initials. Localisation of PIN3 protein is seen all around the cell rather than in a polar fashion. In the *pin3* mutant the columella cells do not differentiate

until they are further from the in the position of the second row of cells, which forms the second row of differentiated columella in the wild type. This suggests that for the change in cell fate to occur from columella initial to columella root cap requires specific and directed auxin transport. It seems to be maintaining the concentration of auxin within these cells and the cells around them so that they can differentiate properly (Friml *et al.*, 2002b).

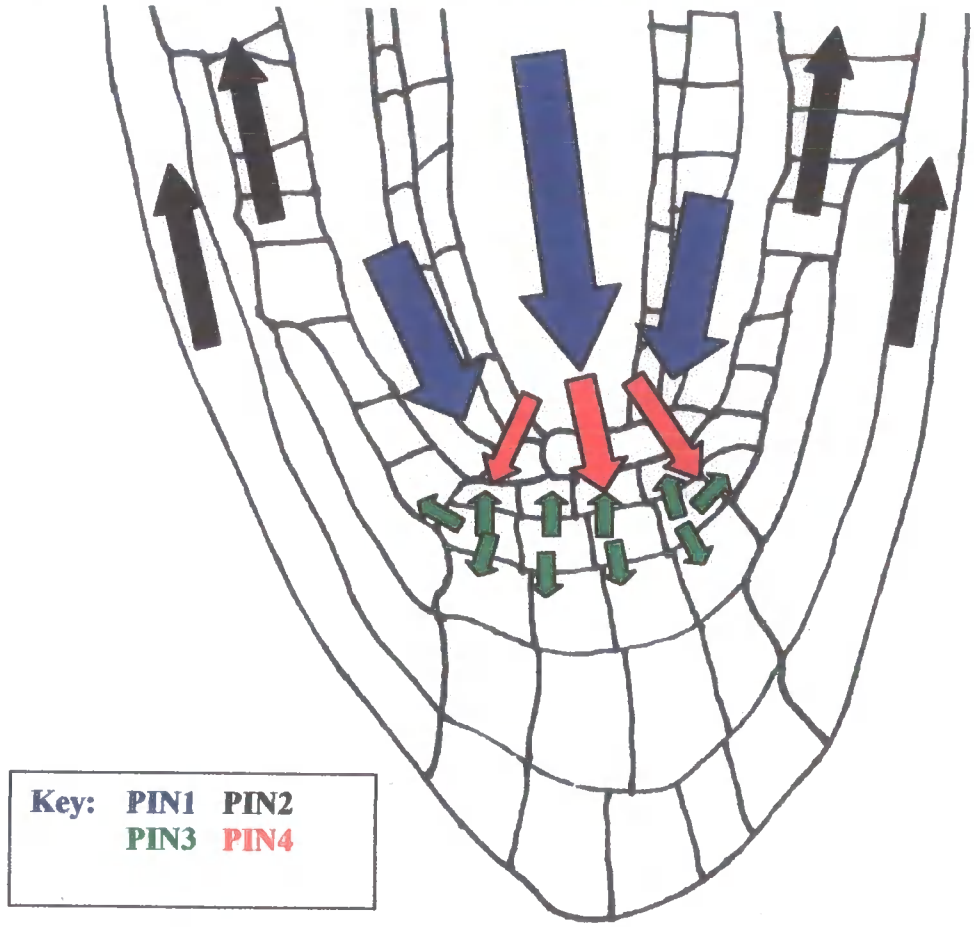
A role for auxin in patterning the root tip has been suggested from studies using the synthetic auxin reporter *DR5* (Sabatini *et al.*, 1999), and the identification of *PIN3* and *PIN4* supports this model. *DR5* GUS activity shows a 'maximum' in the distal root tip, in the initials. In *pin3* mutants the 'maximum' is seen more distally in the root tip (J. Friml, personal communication), whilst in the *pin4* mutants it is seen further back in the stele, behind the meristem (Friml *et al.*, 2002a). *PIN4* is expressed in the root meristem, covering the quiescent centre and the initials, directing auxin from the vascular stele across the root meristem. This is consistent with a role for PIN4 in generating a sink of auxin in the root tip, and the auxin 'maximum' that is important in generating the pattern of root tissues (Sabatini *et al.*, 1999), which is them redistributed by other PIN proteins, such as PIN2, throughout the root (Friml *et al.*, 2002b).

Figure 1.2.1.3 summarises the position of *PIN* proteins within the cell layers of the root, and how they direct the auxin transport stream through the root tip.

Direct Evidence?

However good this indirect evidence is, so far no data has been forthcoming on the binding and transport efficiency of these proteins, both AUXs and PINs. This has mainly been due to problems expressing a membrane-bound protein within systems that are amenable to transport kinetics studies, such as bacteria, animal and insect cell lines (R. Swarup, personal communication, and O. Tietz, personal communication). But, when *PIN2/AGR1/EIR1* was expressed in *Saccharomyces cerevisiae* it conferred an increased resistance to F-IAA, a toxic IAA derivative, and a decreased ability to retain pre-loaded ^{3}H-IAA (Chen *et al.*, 1998; Luschnig *et al.*, 1998). However no kinetic data is as yet available.

Figure 1.2.1.3 PIN Proteins and Auxin Transport in the Root



1.2.1.3 How is the Auxin Signal Perceived and Turned into a Response?

Auxin is well known for eliciting a number of different physiological responses and it is in trying to understand this complexity of response that a number of workers have tried to understand how the auxin signal is perceived and transduced. It appears that there are different levels of competency that a cell or even a tissue can have, with respect to their responsiveness to auxin. We are still a long way off from seeing the whole picture, but a lot of evidence has been emerging in the last few years, thanks again to the completion of the *Arabidopsis* genome project.

Auxin can be perceived at different levels, both at the cell surface as well as within the cell, and even inside the nucleus. It seems that different physiological responses are

perceived in certain places, leading to more specific signal transduction pathways bringing about specific responses.

There are thought to be receptors at the cell surface as well as in the cell itself, particularly in the nucleus. Auxins are lipophilic weak acids that are freely able to permeate the plasmamembrane, so a response cascade could originate from auxin perception within the cell (Lazarus *et al.*, 1991). Also, not all responses need emanate from the same hormone-receptor interaction, as receptors initiating diverse responses could have different subcellular locations and/or different structures (Lazarus *et al.*, 1991). Transduction pathways in the cell transmit the signal from the receptor molecule on the cell surface to the nucleus, where the response is often initiated through a change in gene expression.

An Auxin Receptor - *AUXIN BINDING PROTEIN1*

Many mammalian hormone systems require the binding of the hormone to a receptor molecule. *AUXIN BINDING PROTEIN1* (*ABP1*) is a protein with auxin binding activity that was purified from maize coleoptiles some 20 years ago (reviewed in Klambt , 1990, and Jones, 1994). It has no counterpart in mammals yet has been identified in a number of plant species, including *Arabidopsis*, poplar, tobacco and radish (Jones, 1994). The maize *ABP1* was the first plant protein to be found to have a KDEL sequence, an endoplasmic reticulum retention signal (Pelham, 1988), yet it has been shown that *ABP1* has a low affinity for auxins at the pH of the endoplasmic reticulum (Tian *et al.*, 1995), which rules this out as the location of auxin binding. However, immunolocalisation studies have shown that there a detectable amount of the protein at the plasma membrane (Jones and Herman, 1993), estimates suggest that some 1000 molecules are present at the membrane surface, just 2% of the cell's total complement (Diekmann *et al.*, 1995). However *ABP1* does not contain any hydrophobic domains and so it must associate with another membrane-bound protein, possibly a docking protein, but this has not been identified yet.

The three-dimensional structure of *ABP1* holds the key to understanding how it binds auxin and then propagates an output signal. So far computer modelling has proven the most accessible approach upon which to start to develop models of auxin binding and signal generation, although recent reports of crystallisation of *ABP1* for X-ray diffraction analysis mean that the accuracy of the computer modelling will soon be

tested (Woo *et al.*, 2000). Based on its similarities with proteins of the cupin and vicilin superfamily, modelling of the binding site suggests that *ABP1* forms a β-barrel structure with a cluster of amino acids that could bind a metal ion at its centre. IAA is a small molecule with a planar indole ring system and a carboxylic acidic side chain. The metal ion could complement the carboxylic acid group of IAA whilst an adjacent tryptophan would provide a hydrophobic platform for its indole ring (Warwicker, 2001). Binding of IAA therefore displaces the carboxyl-terminus of *ABP1* from the auxin-binding site forcing a conformational change in the protein, and allowing the propagation a signal by the mobile carboxyl-terminus, possibly through interactions with other proteins. Early X-ray analysis confirms the presence of a β-barrel (Woo *et al.*, 2000), whilst differential binding of antibodies raised to portions of unbound *ABP1* confirm a conformational change in the protein when auxin is bound, and support this model (Napier and Venis, 1990; David *et al.*, 2001). Once purer crystals of *ABP1* are available, in both conformations, then more detail of the binding site and the conformational change can be determined.

The absence of transmembrane domains within *ABP1* has led to the generation of a model involving a membrane-associated docking protein, that may be exported with *ABP1* from the endoplasmic reticulum, or may already be present before *ABP1* reaches it. There is some evidence that this docking protein may be a member of the G-protein-coupled receptor (GPCR) family (Zania *et al.*, 1990; Milner *et al.*, 1996). These receptors typically contain seven transmembrane domains and are coupled to heterotrimeric G-proteins, which consist of an α subunit that binds guanine nucleotides, and β and γ polypeptides which form a single functional subunit. When inactive, GDP is bound to the a subunit which is associated with the by subunit and the receptor. Activation of the receptor, in this case through auxin binding to *ABP1* and causing a conformational change that activates the receptor, causes a change in the α subunit, reducing its affinity for GDP and allowing GTP to bind. The now activated α and βγ subunits dissociate from the receptor and from each other, and act on their effectors, which causes the hydrolysis of GTP to GDP, whereupon the subunits re-associate with the receptor (Macdonald, 1997).

A GPCR is currently a good candidate for the docking protein, however very little is known about the downstream targets of the heterotrimeric G-proteins as well as any further downstream events.

A growing amount of evidence has hinted at *ABP1*'s role in certain auxin responses; specific antibodies generated to *ABP1* alter auxin sensitivity (Barbier-Brygoo *et al.*, 1990), and over-expression of *ABP1* in tobacco leaves causes an increase in auxin induced cell expansion (Jones *et al.*, 1998). However the current discrepancies between its site of action and mode of action are major stumbling blocks to our understanding of how *ABP1* functions in mediating auxin responses *in planta*. The embryo lethality of the *abp1* knock-out mutation in *Arabidopsis* indicates a crucial role for *ABP1* in growth and development (Chen *et al.*, 2001), and hopefully new molecular approaches will help to produce a clearer picture of its role in which responses it initiates and those that it does not.

AUX/IAAs – Active Repressors of Early Auxin Genes

Early auxin response genes were found to be induced by the protein synthesis inhibitor cycloheximide, which suggested the presence of a short-lived nuclear repressor (Ballas *et al.*, 1993, 1995). *AUX/IAA* genes are rapidly induced by auxin, with transcripts detectable within 4-30 minutes of application, and code for short lived (6-8 minute half-life) low abundance nuclear localised proteins. They show a high degree of sequence similarity in four domains (I-IV) but otherwise are highly divergent in both size (25-36 KD) and composition. Domain II is involved in regulating the stability of the protein, and mutations in domain II have been shown to result in an increase in the life span of the protein (Ouellet *et al.*, 2001; Worley *et al.*, 2000). Domain III contains DNA binding domains that are similar to those found in Arc and MetJ repressor proteins (Abel *et al.*, 1994). Together, domains III and IV are involved in homodimerisation between other AUX/IAA proteins, as well as heterodimerisation with a class of transcription factors known as ARFs (Auxin Response Factors). There are twenty-nine *AUX/IAA* genes present in *Arabidopsis* (Liscum and Reed, 2001), and complexity of response is possibly achieved through the numerous combinations of AUX/IAAs and AFRs.

A number of mutations have already been identified in a number of *AUX/IAA* genes, such as *shy2*, *axr2*, *axr3*, *bdl* and *iaa28*, which represent mutations in domain II of

IAA3, *IAA7*, *IAA17*, *IAA12* and *IAA28* respectively (Rouse *et al.*, 1998; Tian and Reed, 1999; Nagpal *et al.*, 2000; T. Hamman, personal communication; Rogg *et al.*, 2001). Meanwhile, Theologis and co-workers are using a reverse genetic approach in *Arabidopsis* to identify all the members of the *AUX/IAA* family, and to then find corresponding mutants in order to investigate the functional roles of each AUX/IAA through multiple mutant genetic studies as well as other molecular approaches, such as the yeast-two-hybrid approach.

ARFs and AREs

ARFs are able to form heterodimers with AUX/IAA proteins because they share domains III and IV at their amino-terminal end (Guilfoyle *et al.*, 1998). At the carboxyl-terminal end the ARFs have a domain that is similar to the DNA binding domain of the maize *VP1* transcription factor. ARFs act together as dimers, with specific pairs predominantly forming, such as ARF1 and ARF2, or ARF5 and ARF7 (Tiwari *et al.*, 2001), and bind to specific sequences within the promoters of genes that are auxin up-regulated. In particular TGTCTC has been shown to be sufficient for *ARF* binding, and so is referred to as an Auxin Response Element (ARE) (Guilfoyle *et al.*, 1998).

Binding of ARFs to AREs therefore requires the removal of the repressors, the AUX/IAA proteins, which repress transcription by forming heterodimers with the ARFs through domains III and IV.

How Downstream Genes are Activated – a Model

The auxin response is mediated by the level of auxin within the cell, as a basal level will exist, which causes the transcription of the AUX/IAA proteins. The AUX/IAA proteins then form heterodimers with ARF proteins (dependent on domains III and IV), stopping them from activating the transcription of downstream genes by preventing their binding to AREs. When the cellular level of auxin increases above a certain level, the negative acting AUX/IAA proteins are made unstable and are subsequently degraded (dependent on domain II),. This allows the ARFs to form homodimers and drive transcription of downstream genes with AREs within their promoters.

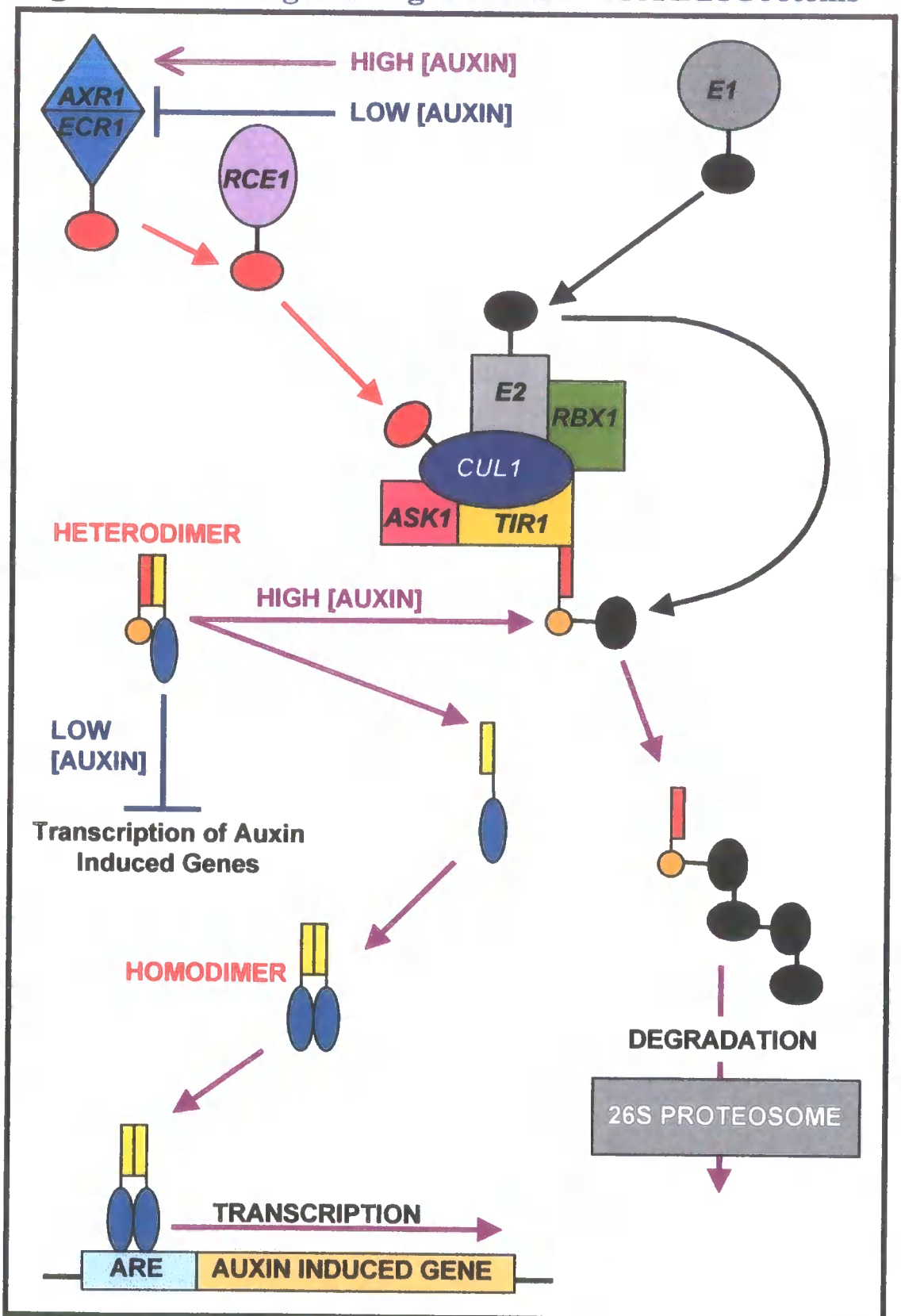
Targeted Degradation of AUX/IAA Proteins

This model therefore requires the targeted degradation of the AUX/IAA proteins in response to auxin, so that the repression of transcription of downstream gene targets can be lifted. Within domain II of the AUX/IAAs is a ubiquitin binding site (Tiwari *et al.*, 2001). Ubiquitin is a 76 amino acid polypeptide that is added to the substrate protein either at several sites within the protein or as a chain of ubiquitin subunits. A protein with a ubiquitin chain of at least four subunits is recognised by the 26S proteosome and degraded (Hershko and Ciechanover, 1998). Ubiquitin-mediated protein degradation plays a critical role in the regulation of diverse biological processes in eukaryotes (Hershko and Ciechanover, 1998), and recently a combination of genetic and molecular approaches in *Arabidopsis* has resulted in the identification of other components of the ubiquitin proteolytic system functioning in the auxin response pathway.

Mutations in either *AXR1* or *TIR1* result in decreased auxin response and a variety of auxin-related growth defects. The *AXR1* gene encodes a subunit of a RUB-activating enzyme related to the E1 ubiquitin-activating enzyme (Leyser *et al.*, 1993; del Pozo *et al.*, 1998), whilst the *TIR1* gene encodes an F-box protein that is a component of an E3-ubiquitin ligase *SCF* complex (Gray *et al.*, 1999). The identification of mutations within these two components led to reverse genetic approaches for other components of the these complexes that were known to be part of other systems. From this work, a convincing model has emerged, which is shown in Figure 1.2.1.3 (adapted from Ward and Estelle, 2001; Leyser, 2001; Gray *et al.*, 2001).

In response to high cellular auxin concentrations, the negative acting AUX/IAAs are targeted for degradation by the ubiquitin ligase SCF^{TIR1} complex, thus releasing the ARF proteins to activate downstream transcription of early response genes. The SCF^{TIR1} complex is activated by an *AXR1*-dependent RUB modification of *CUL1*; RUB is a protein that is related to ubiquitin. *AXR1* and *ECR1* form a heterodimer, passing RUB onto the RUB-conjugating enzyme, coded for by *RCE1*, which then activates *CUL1* by conjugating it with RUB. *CUL1* is a component of the E3-ubiquitin ligase *SCF* complex along with a Skp-I like gene (*ASK1*), *RBX1*, and an F-box protein (*TIR1*) (Gray *et al.*, 1999).

Figure 1.2.1.3 Targeted Degradation of *AUX/IAA* Proteins



Adapted from Ward and Estelle (2001), Leyser (2001) and Gray *et al.*, (2001).

Once activated, ubiquitin is delivered by the E1 ubiquitin-activating enzyme to the E2 ubiquitin-conjugating enzyme, which is also part of the SCF^{TIR1} complex. As well as initiating the RUB activation of SCF^{TIR1}, auxin also stimulates the interaction between *TIR1* and domain II of the AUX/IAA proteins, resulting in the ubiquitination of the *AUX/IAA* protein, which is targeted for degradation by the 26S proteosome (Gray *et al.*, 2001).

During this lifting of repression, AUX/IAA proteins are still transcribed and translated, as their transcription is activated by auxin. This means that when the level of auxin drops, the AUX/IAA proteins are ready to actively repress transcription, including their own transcription, until auxin levels increase such that it activates the RUB modification of the SCF^{TIR1} complex and causes their degradation (Tiwari *et al.*, 2001).

1.2.2 Ethylene

Ethylene (C_2H_4) is a simple gaseous hydrocarbon molecule that has a number of important effects on plant development, such as in fruit ripening, leaf senescence, flower abscission and seed germination. It is also involved in a range of stress responses; flooding, wounding or pathogen attack. C_2H_4 also has roles in tissue patterning within plant development (Tanimoto *et al.*, 1995), as well as a putative role in regulating auxin transport (Luschnig *et al.*, 1998). Ethylene, being gaseous, can therefore act both as an intercellular signal as well as an intracellular signal.

The physiological activity of ethylene gas was first noted through the ability of smoke and coal gas to induce flowering, fruit ripening, and leaf abscission (Neljubov, 1901; Kieber, 1997). It was first found to be released by naturally ripening fruit, but is now known to be produced by most plant tissues, particularly as a stress response as well as in maturing and senescing organs. Ethylene evokes a wide range of responses, such as fruit ripening, leaf and fruit abscission in dicotyledonous species, flower senescence, stem extension in aquatic plants, gas space (aerenchyma) development in roots, and epinastic curvature in flooded plants.

As well as developmental responses, ethylene is involved in stress responses, and it would therefore seem necessary to ensure that it does not activate the wrong response. It

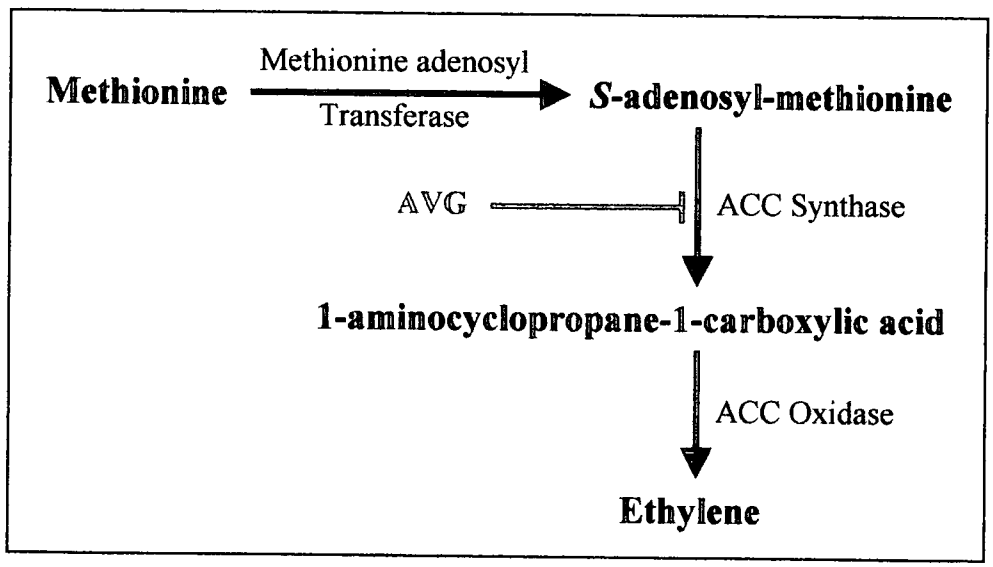
is a matter of opinion as to whether all ethylene responses can be regarded as stress responses, but they all seem to help the plant in adapting to its environment.

The stress responses can be seen in the production of aerenchyma cells in flooded plants, and in the triple response, which protects the developing seedling as it pushes through the soil. The triple response results in a reduced rate of root and shoot elongation, a thickening of the hypocotyl, and the exaggerated apical hook, which protects the apical meristem from mechanical damage.

1.2.2.1 Ethylene Biosynthesis

Ethylene can be synthesised by most cell types, indeed it has not been localised to a specific area like auxin. Ethylene is produced from methionine, which is converted to *S*-adenosyl-methionine by methionine adenosyl transferase, which is then in turn converted to 1-aminocyclopropane-1-carboxylic acid (ACC). ACC can be transported throughout the plant by the vascular tissue, and then once off loaded, converted locally into ethylene. It is made from *S*-adenosyl-methionine by the *ACC SYNTHASE* (*ACS*) genes, a multi-isozyme family that shows precise temporal and spatial expression patterns. Different members have specific stimuli for their production, such as auxin, cytokinin and wounding. ACC synthase is the rate limiting enzyme in the pathway (Liang *et al.*, 1995).

Figure 1.2.2.1 Ethylene Biosynthesis



The next enzyme in the pathway converting ACC to ethylene is ACC oxidase. It appears that this family of enzymes are constitutively expressed throughout all tissues, suggesting that it is the expression of *ACS* genes in space and time that determines where and how much ethylene is to be produced. It appears that ethylene's unique role within the different plant responses is the product of much of it's biosynthesis and transport.

Applying exogenous ethylene to plants is very difficult to do, and requires specialised equipment and may produce non-localised increases in ethylene. Therefore ACC is often used in the growth medium, as when it is taken up it increases the production of ethylene in the plant. AVG can be used to inhibit the action of ACC synthase enzymes (Lieberman *et al.*, 1975; Tanimoto *et al.*, 1995), whilst silver ions (silver nitrate, $AgNO_3$) inhibits ethylene perception at the receptor (Beyer, 1976).

1.2.2.2 Ethylene Perception and Signal Transduction

Ethylene is perceived by a family of receptors that are similar to bacterial two-component histidine kinase receptors, which consists of two subfamilies. Both subfamilies contain an amino-terminal ethylene binding domain followed by a 'GAF'-related domain (Aravind and Ponting, 1997) and a histidine protein kinase domain (Chang *et al.*, 1993). However only certain members of each subfamily contain the second part of the two-component system, a carboxyl-terminal receiver domain. In *Arabidopsis* there appear to be five ethylene receptors within the genome, with *ETR1* and *ERS1* making up the first subfamily, and *ETR2*, *EIN4* and *ERS2* making up the second (Chang and Shockey, 1999).

The most conserved region in all of the family members is the amino-terminal ethylene binding domain, which was found to be sufficient for reversible ethylene binding in *ETR1* and *ERS1* (Schaller *et al.*, 1995; Rodriguez *et al.*, 1999), and is expected to confer the same ability to members of the *ETR2* group because of similarity in both sequence and mutant phenotype (Sakai *et al.*, 1998; Hua *et al.*, 1998; Hua and Meyerowitz, 1998). Dominant mutations conferring ethylene insensitivity have been isolated in *ETR1*, *ETR2* and *EIN4* (Chang *et al.*, 1993; Sakai *et al.*, 1998; Hua *et al.*, 1998), each causing a mutation within one of the three amino-terminal membrane-spanning

segments, and when identical mutations have been introduced into *ESR1* and *ESR2* they have caused a similar ethylene insensitivity (Hua *et al.*, 1995; Hua *et al.*, 1998).

ETR1 has been shown to be a membrane-bound disulfide-linked homodimer, that has two copper ions per homodimer to allow high-affinity ethylene binding (Schaller *et al.*, 1995; Rodriguez *et al.*, 1999). Silver ions have long been known to inhibit ethylene action in non-competitive manner, and it was originally thought to block ethylene binding by associating with the copper co-factor (Beyer, 1976). However recent studies have shown that silver ions actually enhances ethylene binding, suggesting that it blocks ethylene signalling downstream of ethylene binding (Rodriguez *et al.*, 1999).

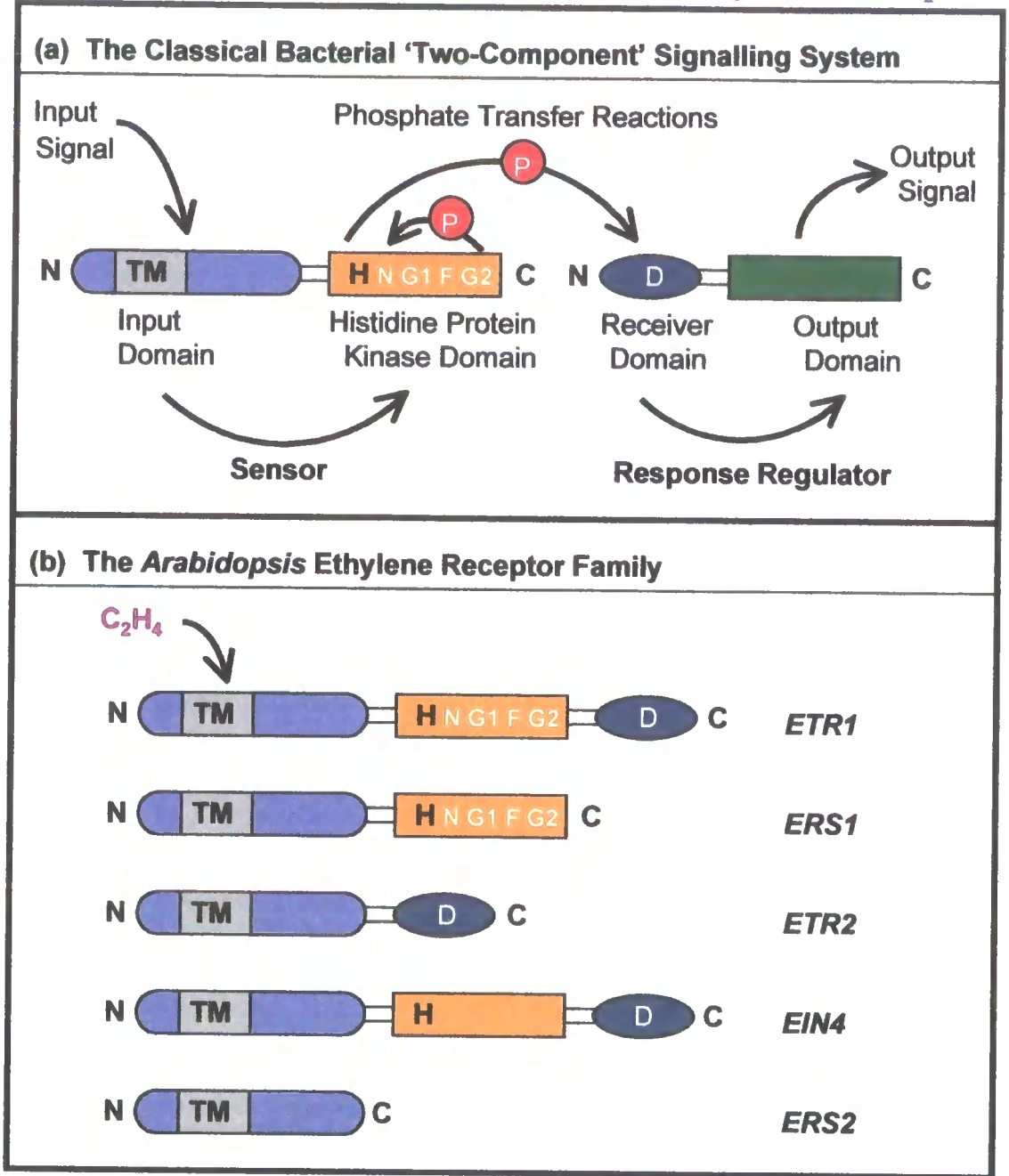
So far the most detailed studies of the whole family have been carried out in tomato, where all five members showed distinct developmental expression patterns, indicating that the receptors have different tissue- and stage-specific roles in regulating ethylene signalling, and that ethylene responses may be regulated at the receptor level (Lashbrook *et al.*, 1998; Tieman *et al.*, 1999; Zhou *et al.*, 1996; Payton *et al.*, 1996).

Signal Transduction

Following the bacterial two-component signalling model, the binding of ethylene to the receptor would induce a phosphotransfer from a histidine residue in the histidine autokinase domain to an aspartate residue in the receiver domain of the response regulator component (Parkinson and Kofoid, 1992). Figure 1.2.2.2 shows the major components of this system and how it relates to each member of the *Arabidopsis* receptor family.

Receptor loss of function mutants confer ethylene insensitivity, and when mutations in *ETR1*, *ETR2* and *EIN4* are combined, constitutive ethylene responses result, indicating that the receptors normally act to repress responses in the absence of ethylene (Hua and Meyerowitz, 1998). Binding of ethylene therefore shuts off receptor signalling, which consequently activates the downstream ethylene response pathways. Interestingly, mutations in the *RAN1* gene, which codes for a P-type ATPase that delivers copper ions from intracellular storage sources to copper-utilising enzymes, develop a constitutive ethylene-response phenotype similar to the multiple receptor loss-of-function mutants (Hirayama *et al.*, 1998; Woeste and Kieber, 2000).

Figure 1.2.2.2 The Ethylene Receptor Family of *Arabidopsis*



(a) Bacterial 'two-component' signalling systems are made up of distinct modules that can be arranged in different ways in multi-domain proteins - in the archetypal systems, for example, the 'sensor' and 'response regulator' are separate proteins. Information flows from one module to another by a combination of non-covalent interactions and phosphorylation reactions involving histidine (H) and aspartate (D) residues. (b) The *Arabidopsis* ethylene receptor family have an ethylene binding site in the transmembrane domain (TM), followed by a combination of each of the component modules. Only *ETR1* and *ERS1* have the conserved motifs (N, G1, F and G2) that are considered necessary for histidine kinase activity.

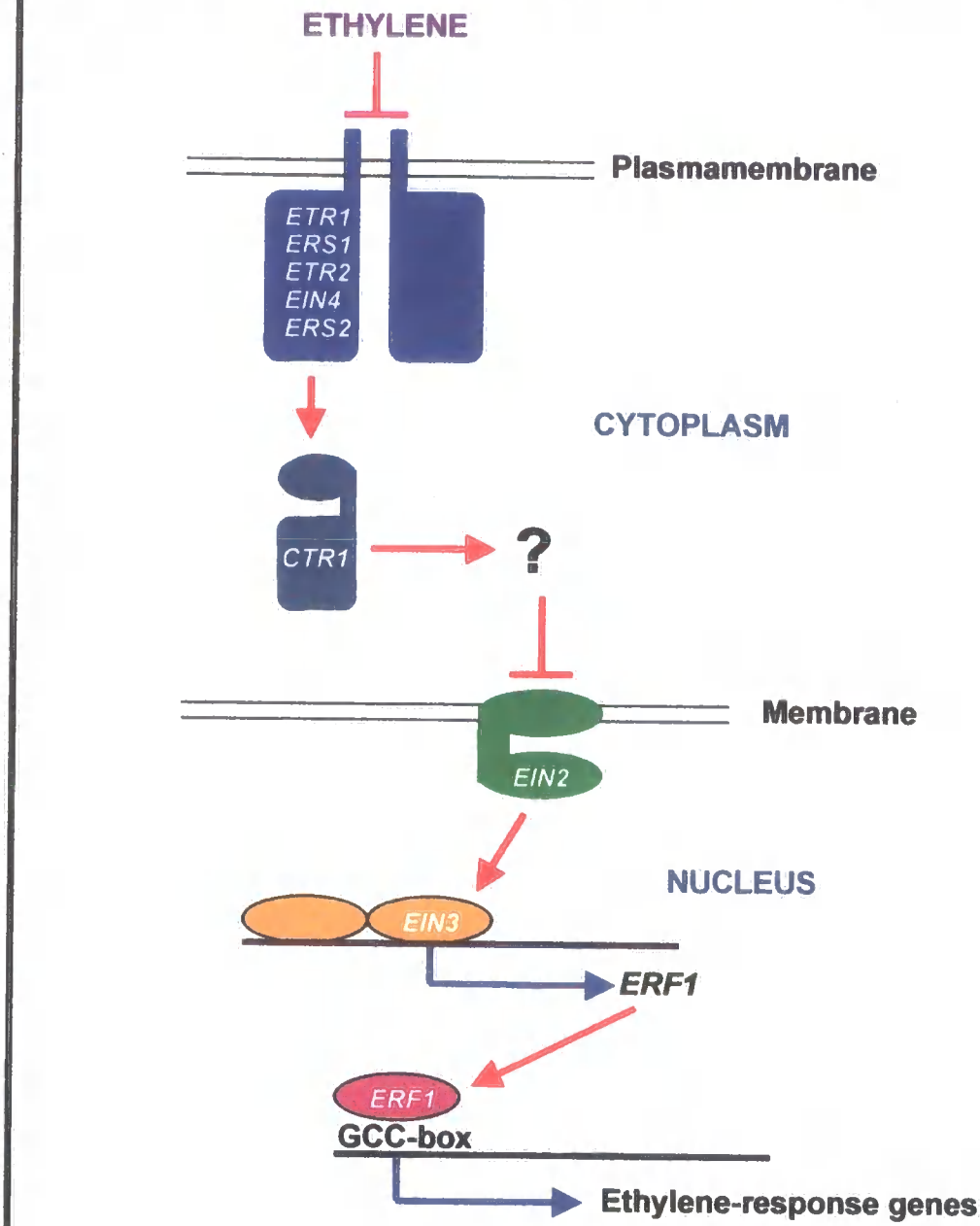
Figure constructed from Theologis (1996) and Chang and Stadler (2001).

The next known component of the pathway that is downstream of the receptors is *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*), which is similar to the Raf family of mitogen-activated protein kinase kinase kinases (MAPKKK). A direct interaction between the amino-terminal domain of *CTR1* and the histidine kinase domain of both *ETR1* and *ERS1* has been shown by yeast two-hybrid assay and by *in vitro* co-purification techniques (Clark *et al.*, 1998). However it is not known whether this interaction is sufficient to transfer the signal from the receptors to *CTR1*, although the recent three-dimensional modelling of the *ETR1* receiver domain shows structural similarity to Ras proteins, which are involved in activating Raf kinases (Mueller-Dieckmann *et al.*, 1999). Both Ras and Raf are known to interact and therefore signalling between *ETR1* and *CTR1* could be mechanistically similar. More work in this area is required to not only confirm the details of this interaction, but also to identify other proteins and possibly other pathways through which the ethylene receptors interact with downstream targets.

Altering Gene Expression

Downstream of *CTR1* is *ETHYLENE INSENSITIVE2* (*EIN2*), which is one of the central players in the ethylene signal transduction pathway as well as other pathways. *ein2* mutants show high ethylene insensitivity, and of the 25 alleles isolated, some were found in screens for resistance to auxin transport inhibitors, and insensitivity to cytokinin or ABA. No other ethylene loci have been isolated in these screens, suggesting that it is a central component of other signalling pathways (Alonso *et al.*, 1999). The function of the *EIN2* protein is difficult to interpret because as it has a number of interesting domains. The amino-terminus has twelve membrane spanning domains, suggesting that it anchors the protein, and has similarity to the Nramp family of ion transporters, however no metal transporting activity has been noted so far. The amino-terminal has also been shown to have an autoinhibitory domain. The carboxyl-terminal domain has a coiled-coil structure which suggests a role in protein-protein interactions. The ethylene signal could be therefore be sensed at the amino-terminal end, possibly through an unknown divalent cation acting as a second messenger, whilst the carboxyl-terminal end could represent the activation domain, which could be brought into contact with the correct component by activation of the amino terminal domain. Therefore implying a two component activity on *EIN2*, acting as a bifunctional transducer (Alonso *et al.*, 1999).

Figure 1.2.2.3 Model of Ethylene Signal Transduction



Schematic diagram of the ethylene signal transduction pathway in *Arabidopsis*. Five ethylene receptors (*ETR1*, *ERS1*, *ETR2*, *EIN4*, and *ERS2*) relay the signal to the *CTR1* protein kinase, which is a negative regulator. *CTR1* is presumed to be the first component in a MAP kinase cascade (which is indicated by "?"). *EIN2*, which acts downstream of *CTR1*, has an integral membrane domain, however its specific location is not yet known. Downstream of *EIN2*, a novel transcription factor called *EIN3* promotes transcription of a second transcription factor, *ERF1*. *ERF1*, in turn, binds to the GCC-box promoter element to activate transcription of specific-ethylene response genes. Taken from Chang and Stadler (2001).

The *EIN3* gene codes for a nuclear-localised DNA binding protein that acts down stream of *EIN2*. Three other members of this family have been found in *Arabidopsis*, named *EIN3-LIKE* (*EIL*), and over expression of *EIN3* or *EIL1* results in constitutive ethylene responses (Chao *et al.*, 1997). *EIN3* has been shown to bind to a conserved *cis*-element in the promoter of an EREBP-encoding gene called *ETHYLENE RESPONSE FACTOR1* (*ERF1*) (Solano *et al.*, 1998). *ERF1* in turn binds to a *cis*-acting sequence known as the GCC-box, which is located in the promoters of secondary target genes, therefore acting as a transcriptional activator (Solano *et al.*, 1998; Hao *et al.*, 1998). Over expression of *ERF1* leads to the same constitutive ethylene responses as *EIN3* and *EIL1* over expression.

Ethylene signalling therefore involves a transcriptional cascade that allows amplification of the signal to activate ethylene response genes. Figure 1.2.2.3 shows a model for ethylene signalling.

1.2.3 Cytokinin

Cytokinins, which are N^6-substituted adenine derivatives, were first described as promoters of cell division in the presence of auxin by Miller and co-workers (1955). Kinetin (6-furfurylaminopurine) was the first to be described and was isolated from autoclaved herring sperm DNA, and so is not of a natural plant product. Natural cytokinins include zeatin, dihydrozeatin, isopentenyladenine and benzyladenine, and they play a central role in controlling cell division and cell fate (Mok and Mok, 1994) As well as their activity in tissue cultures they have diverse effects in intact plants, including axillary bud outgrowth, delay of leaf senescence, and control of nutrient metabolism.

The lack of mutants within the cytokinin biosynthetic pathway has meant that much study has required either genetic manipulation with bacterial genes or radioactive feeding experiments. The biological significance of many enzymes in both the synthetic and degradative pathways therefore is unclear. The root tip has been identified as a major site of cytokinin biosynthesis and are assumed to be transported in the xylem to exert their effects in the shoot; cytokinins have been identified in xylem sap (Haberer and Kieber, 2002). Immunolocalisation and direct measurements of

cytokinin have shown high levels accumulating in mitotically active areas such as the shoot and root meristem (Mok and Mok, 1994; Dewitte *et al.*, 1999).

1.2.3.1 Cytokinin Mutants

A number of mutant screens have produced cytokinin insensitive mutants. *cytokinin resistnat1* (*cyr1*) was the first to be identified with specific insensitivity to cytokinin (Deikman *et al.*, 1995). Root elongation in this mutant is sensitive to inhibition by auxins, ethylene and abscisic acid, but is resistant to inhibition by cytokinin. The mutants also have limited shoot growth, with reduced cotyledons and leaf expansion, and produce one single infertile flower.

The *cytokinin insensitive* mutants (*cin1 – 5*) were isolated from a screen for plants that failed to induce a triple response when grown on low levels of cytokinin in the dark (Cary *et al.*, 1995). The triple response is mediated by ethylene, and so when *CIN5* proved to be an ACC synthase gene (*ACS5*), it was no surprise (Vogel *et al.*, 1998). However the link with cytokinin was not fully understood, and the isolation of this mutant has shown that cytokinin mediates some of its effects through the up-regulation of *ACS5*. This gene is post-transcriptionally regulated by cytokinin. *CIN1*, *CIN2* and *CIN3* appear to be genes involved in ethylene production through both cytokinin and other stimuli, and act upstream of *ACS5* (Vogel *et al.*, 1998).

1.2.3.2 Bacterial Two-Component Receptors and Cytokinin Signal Transduction

As we have already discussed, two-component receptors are a key feature of ethylene signalling, and it was with much surprise that their role in cytokinin signalling was discovered.

The *cytokinin independent* (*cki*) mutant was isolated from an activation tag T-DNA population for constitutive cytokinin responses in the absence of cytokinin, such as cell proliferation and shoot formation in calli (Kakimoto, 1996). When cloned, the *CKI1* gene was found to produce a protein that possesses an input domain, a histidine kinase domain and a receiver domain, similar to *ETR1* and the ethylene receptor family. In

addition to these features it also has two extra membrane spanning domains at the amino terminus (Kakimoto, 1996). It is therefore attractive to hypothesise that *CKI1* encodes a cytokinin receptor, however no functional binding of cytokinin has been shown in the same way that ethylene has been shown to bind to the *ETR* receptors. Once knock-outs have been isolated and more functional analysis carried out, it will be easier to see how this protein fits into the pathway within the whole plant in space and time.

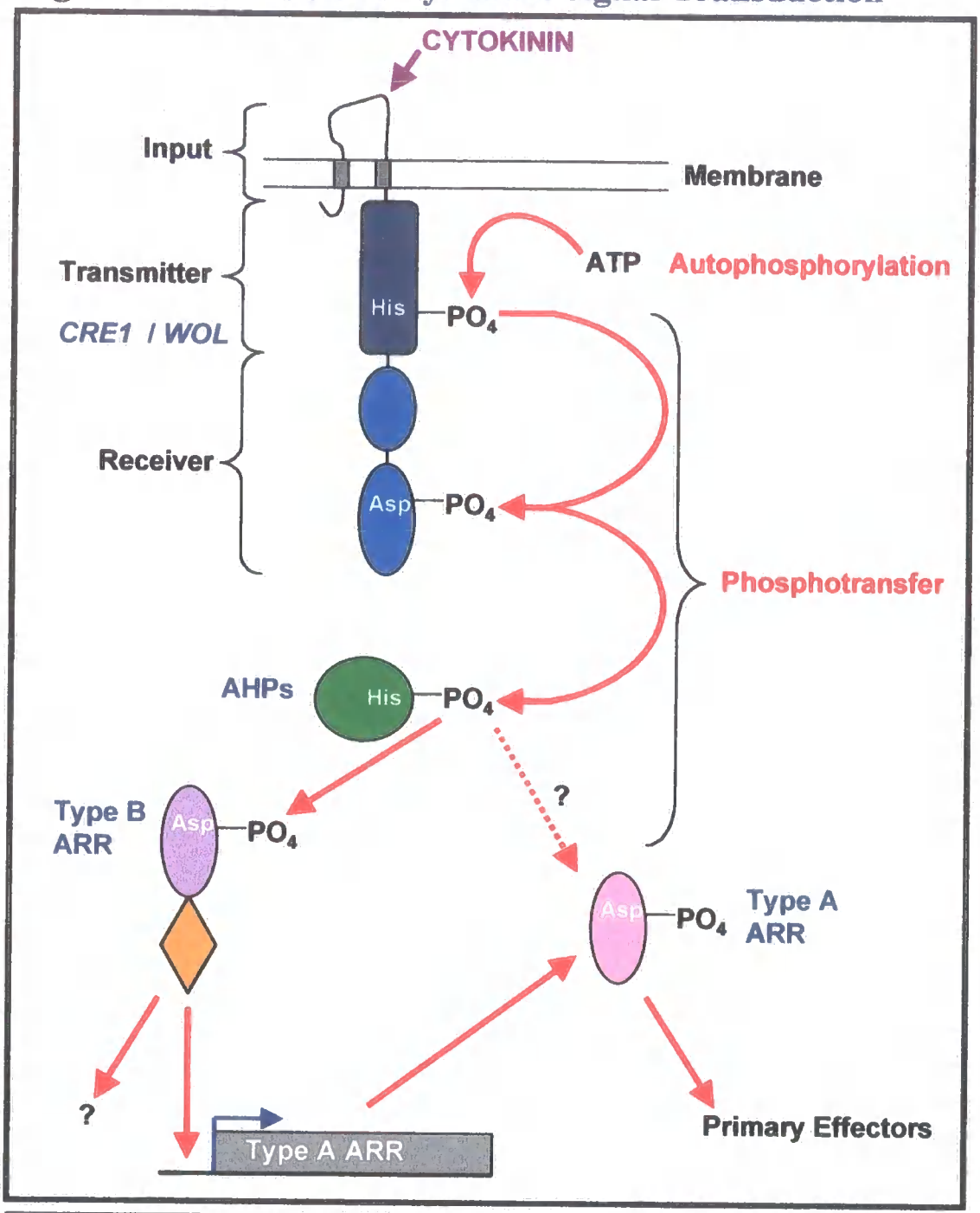
The *cytokinin response* (*cre1*) mutant was isolated from a screen for impaired cytokinin responses. *CRE1* was found to encode a two-component histidine kinase (Inoue *et al.*, 2001), adding further weight to a model of phosphorelay signalling in cytokinin perception and signal transduction. Furthermore, *Arabidopsis* has two homologues, *AHK2* and *AHK3*, that share high sequence homology with *CRE1*. These two other homologues may also function in cytokinin signalling, and may account for the less severe phenotype of the *cre1* mutants, as some functional redundancy may exist. It will therefore be important to uncover the tissue specific and developmental specific roles of these three proteins in order to see how important they are to cytokinin signalling.

Signal Transduction

The downstream partners of *CRE1* are now being found thanks to the knowledge built up of phosphorelay signalling in yeast and *E. coli*, and the information available from the *Arabidopsis* Genome Initiative. Figure 1.2.3.2 shows a possible model for signalling based on analogy to other sensor kinases. Upon stimulation of the input domain by cytokinin, it could dimerize and autophosphorylate on a histidine residue within the transmitter domain. The phosphate would then be transferred to an aspartate residue on the fused receiver domain and then to a histidine on an HPt domain-containing protein (HP). Finally the phosphate may be transferred to an aspartate residue on the receiver domain of a response regulator protein (RR).

Mutations in His459 or Asp973 in the His-kinase and receiver domain respectively, abolishes *CRE1* activity. Furthermore, heterologous expression of *CRE1* in His-kinase receptor mutant yeast and *E. coli* test systems, mutations in the host HP or RR genes abolished complementation by *CRE1* (Inoue et al., 2001; Suzuki et al., 2001). Together

Figure 1.2.3.2 Model of Cytokinin Signal Transduction



Model of cytokinin signalling in *Arabidopsis*. Cytokinin binds to the N-terminal domain of *CRE1* and activates its His kinase activity. *CRE1* phosphorylates the AHPs, which in turn transfer the phosphate to the receiver domain of B-type ARRs, thus activating their output (transcription activator) domain. Type A ARRs also interact with the AHPs, and are also likely phosphorylated. The activated Type A ARRs, perhaps in parallel and/or in combination with the activated type-B proteins, interact with various effectors to alter cellular function.

Adapted from D'Agostino and Kieber (1999), and Haberer and Kieber (2001).

these results indicate that *CRE1* uses a Hip-Asp phosphorelay typical of His-kinases, and that it utilises a HP protein and RR to bring about changes in gene expression.

Five genes encoding HP-type transmitter proteins are present in *Arabidopsis* (*AHP1-AHP5*) (Suzuki *et al.*, 2000), and roughly 20 RR genes (*ARR*) (Imamura *et al.*, 1998; D'Agostino *et al.*, 1999; Imamura *et al.*, 1999; The *Arabidopsis* Genome Initiative, 2000). The *ARR* genes belong to two classes, Type A and Type B. Transcription of type A *ARR*s is regulated by cytokinin, but as yet have no known function (D'Agostino *et al.*, 2000). Type B *ARR*s are not induced by cytokinin, but possess a myb-related carboxyl-terminal domain and have been shown to act as transcriptional activators (Lohrmann *et al.*, 1999; Sakai *et al.*, 2000).

A current hypothetical model supports a phosphorelay cascade from *CRE1* via *AHP* proteins to the type B *ARR* proteins, which in turn activate the transcription of the type A *ARR* genes, that could activate or repress downstream responses. Consistent with this model is the recent finding that type B *ARR*s are activated through their amino-terminal receiver domain and directly up-regulate the expression of type A ARRs (Schmulling, 2001).

The high number of *ARR*s in *Arabidopsis* allows for a high level of sensitivity of response as they can be regulated in both a temporal and spatial manner. J. Kieber and co-workers have set about an ambitious project to identify mutations in all of the *ARR* genes in *Arabidopsis* and to then create multiple mutant combinations in order to understand the physiological significance of each member. Also, they are looking at *AHP* and *ARR* genes in a yeast two-hybrid system, to determine the specificity of the relationships involved in the phosphotransfer reactions (J. Kieber, personal communication).

1.2.4 Brassinosteroids

In 1970 Mitchell and co-workers isolated a group of growth promoting compounds from pollen extracts of *Brassica napus*, and termed them 'brassins' (Mitchell *et al.*, 1970). Subsequently, Grove *et al.*, (1979) were able to show that this activity was due to brassinolide, a molecule with structural similarity to mammalian steroid hormones.

Since then, over 40 analogues have been identified from a wide variety of plant species, and the biological activity of this group, the brassinosteroids (BRs), has been investigated in more detail. Through the use of molecular genetic approaches and a number of mutant screens, key genes and molecular mechanisms have been identified. This intensive research effort has led to the BRs being accepted members of the group of main endogenous plant growth regulators.

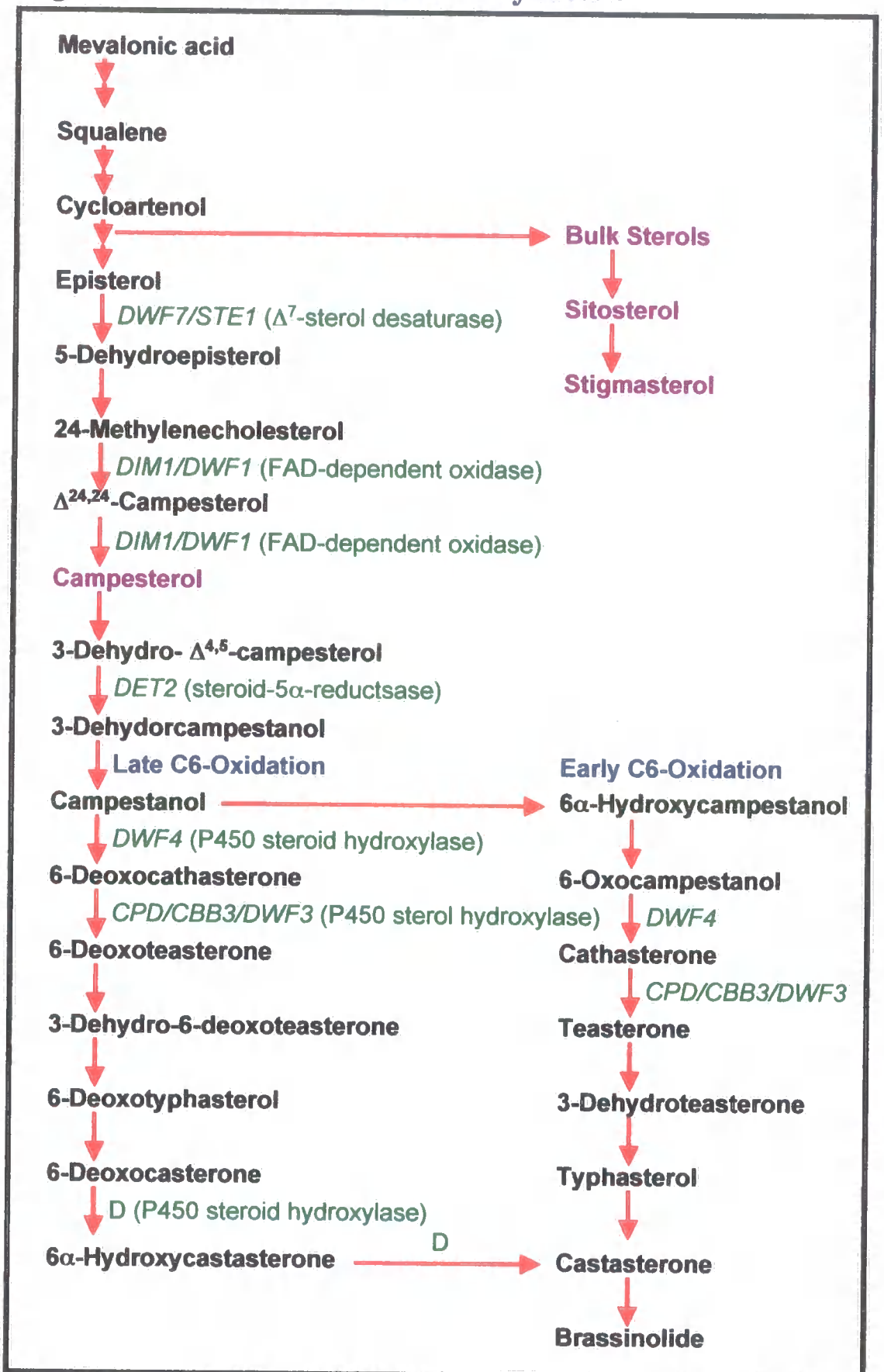
The exogenous application of BRs leads to a spectrum of growth responses, such as stem elongation, inhibition of root growth, leaf epinasty, pollen-tube growth, xylem differentiation, membrane hyperpolarization, enhanced ethylene production and altered endogenous phytohormone levels (Mandava, 1988; Sakurai and Fujioka, 1993; Yokota, 1997; Clouse and Sasse, 1998). Principally though, BRs are believed to stimulate growth through enhanced cell divisions and cell elongation (Mandava, 1988), acting synergistically with auxins (Katsumi, 1985; Kim *et al.*, 1990; Yopp *et al.*, 1981) and additively with gibberellins (Katsumi, 1985; Mandava *et al.*, 1981).

1.2.4.1 BR Biosynthetic Mutants

Several independent mutant screens isolated dwarfed mutants that were rescued to a wild type growth habit when supplied with exogenous brassinolide. The cloning of these loci has led to a clearer elucidation of the biosynthetic pathway through which BRs are produced. Sterols form the precursors for BR production, and as Figure 1.2.4.1 shows, the BR pathway branching off from the production of bulk sterols (sitosterol and stigmasterol) through another bulk sterol, campesterol. The BR specific pathway then splits into two separate chains, the early C6-oxidation path where hydroxylation of the side chains occurs after C6 oxidation, and the late C6-oxidation path in which the hydroxylation of the side chains occurs before position 6 and the B ring is oxidised (Schumacher and Chory, 2000).

Figure 1.2.4.1 also shows the position of the genes that have so far been identified through the isolation of mutants. *DWF7/STE1* is a Δ^7-sterol desaturase that converts episterol to 5-dehydroepisterol (Choe *et al.*, 1999; Gachotte *et al.*, 1995), while *DWF1/DIM1/CBB1* is a FAD-dependent oxidoreductase, converting 24-methylenecholesterol to campesterol (Klahre *et al.*, 1998; Choe *et al.*, 1999). *det2*

Figure 1.2.4.1 Brassinosteroid Biosynthesis



Key: Genes identified through mutant studies in *Arabidopsis*. Bulk membrane sterols. Figure adapted from Schumacher and Chory (2000).

represents a steroid 5α-reductase that converts campesterol to campastanol (Li *et al.*, 1996). Both *cpd/cbb3/dwf3* and *dwf4* are cytochrome P450 proteins that are similar to steroid hydroxylases, catalysing the C-23 and C-22 steps respectively (Mathur *et al.*, 1999).

Cloning of the genes represented by these mutant loci is helping to piece together this complex pathway, and those mutants still awaiting cloning have been presently placed where biochemical analysis suggests the block exists.

1.2.4.2 Where are BRs made?

An understanding of the location of BR production within the plant during development can be gained by looking at the expression of the biosynthetic genes that have already been cloned. *DET2* is constitutively and ubiquitously expressed, whilst *CPD* is expressed in cotyledon and leaf primordia in dark grown seedlings, and in the adaxial parenchyma of expanding leaves in the light. *CPD* is not expressed in the elongation zone of the root or hypocotyl, suggesting that BRs are made in the cotyledons and leaves before being transported to areas active in cell division and expansion. However, feeding experiments with ^{14}C-labelled 24-epibrassinolide in cucumber and wheat has show that there is movement from the roots to the leaves but not in the other direction. There also appears to be a higher turnover rate in the leaves than in the roots (Fujioka and Sakurai, 1997). Several enzymes have been proposed for the turnover of BRs, however none have so far been isolated in either a forward or a reverse genetic screen.

1.2.4.3 Signal Transduction

Despite the numerous genes that have been isolated in the biosynthetic pathway, only one genetic locus has been identified that corresponds to a BR receptor. Indeed 25 *bri1* alleles have been independently isolated, suggesting that either this is the only BR component or that other components are redundant or parts of other pathways, and are therefore lethal. Knock-out *bri1/cbb2* mutants show the same dwarf phenotype of the biosynthetic mutants, however it cannot be rescued by application of exogenous BRs (Noguchi *et al.*, 1999; Kauschmann *et al.*, 1996).

BRI1 encodes a transmembrane receptor kinase, consisting of a putative leucine-zipper motif, 25 leucine-rich-repeats (LRRs) with a 70 amino acid island buried between the 21st and 22nd LRR, and a cytoplasmic kinase domain with serine/threonine kinase activity (Li and Chory, 1997). Such a receptor kinase is not a normal component of the steroid signal transduction pathways studied in animals, where there are normally nuclear receptor proteins that are ligand-dependent transcription factors. Cell surface steroid receptors are present in mammals, however they only mediate non-genomic effects through a protein-phosphorylation cascade. So how does *BRI1* act in plants? LRRs are believed to mediate protein-protein interactions, but the unique feature of *BRI1* is the 70 amino acid island, and the presence of two mutant alleles in this region suggest an important function. 15 of the 19 alleles not found in the LRR regions are found in the kinase domain, which has possible interactions with type 2C phosphatases (KAPP). *BRI1::GFP* protein fusions are localised to the plasmamembrane, and are ubiquitously and constitutively expressed.

The role of the extracellular LRR domain in *BRI1* has been demonstrated by expression of a chimeric receptor, containing the *BRI1* extracellular domain and the protein kinase domain of *Xa21*, in rice cells (He *et al.*, 2000). *Xa21* is a similar LRR receptor-like protein kinase from rice that functions in disease resistance, and treatment of the rice cells expressing the *BRI1yXa21* chimera with BR induced a number of defence-related responses. These results support the hypothesis that the *BRI1* extracellular domain senses BR directly.

***BRS1* – A Carboxypeptidase that Requires BR and *BRI1* for BR signalling**

In order to look for components of the BR signalling pathway, a weak *bri1* allele was subjected to an activation tagging strategy, looking for gain-of-function suppressors of the *bri1* phenotype (Li *et al.*, 2001). A line was identified, called *bri1 suppressor-Dominant* (*brs1*), and investigated further. Cloning showed it to encode a member of the carboxypeptidase D protease family, enzymes that preferentially remove Arg or Lys from the carboxy-terminus of a peptide. *BRS1* also possesses the amino-terminal signal for the secretory pathway that is also common in this family, meaning that it probably acts extracellularly.

The gain-of-function mutation suppresses 2 weak extracellular domain mutants, *bri1-5* and *bri1-9*, but failed to suppress a cytoplasmic domain mutant, *bri1-1* (Li *et al.*, 2001).

When *bri1-5/9 brs1* double mutants were combined with either *dwf4-1* or *det2-101*, both mutants that cannot produce BR, *brs1* was unable to rescue the *bri1* phenotype. Also, missense mutants with the carboxypeptidase activity removed failed to rescue the *bri1* phenotype. Taken together, these results show that *BRS1* action requires BR, a functional *BRI1* protein kinase domain and *BRS1* protease activity.

A Model for BR Sensing

How exactly *BRI1* senses BR is unknown, but two hypotheses have been proposed for the mechanism of interaction between *BRI1* and BR. The first hypothesis holds that *BRI1* binds BR directly (Li and Chory, 1997), and the second hypothesis supposes that *BRI1* senses BR by binding to a secreted steroid-binding protein that directly interacts with BR (Li *et al.*, 2001).

Two putative steroid-binding proteins have been identified in the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000), which contain processing sites making them possible substrates for *BRS1* (Li *et al.*, 2001). It is therefore possible to propose a model in which extracellular *BRS1* activates one of the two possible BR-binding proteins, allowing them to then bind BR and form a BR-BR binding protein complex. This complex then binds to the extracellular domain of *BRI1* and initiates a signal output through the cytoplasmic protein kinase, possibly through interaction with a type 2C phosphatase.

More work is clearly required in all areas of BR biology, in its biosynthesis, its perception and its downstream gene targets. It is hoped that the recent advancements in genetic techniques such as microarray analysis and T-DNA mutant libraries will help to shed more light upon this interesting hormone.

1.2.5 Hormone Manipulation: Exogenous v Endogenous Approaches

Traditionally, studies of hormone function have involved the application of exogenous hormones to the tissue under investigation, with the aim of altering internal hormone concentrations. This method is also used in early investigations into hormone action on wild-type plants, as well as for characterising new mutants. Applying hormones exogenously to a plant by growing on medium with dissolved hormones, is not only comparatively cheap, but is also very simple. However there are some inherent

problems with applying hormones exogenously, such as measuring the levels of free endogenous hormone, and knowing how much of the hormone the plant has actually taken up. Though this can be overlooked if it is used as the initial part of the investigation into the mutant, and the information used to plan for further investigations. Other more resolute methods for changing hormone levels may then be required, but these are often expensive and time consuming, such as plant transformation.

With the development of gene transfer technology it is possible to manipulate the endogenous hormone levels throughout the plant over a specific developmental time period. This helps to eliminate the uncertainties of uptake and turnover that are caused by exogenous applications. However the main advantage of this technology is that through combinations of different transgenes and previously characterised mutants, the interactions of the different hormones can be unravelled. Since hormones affect several developmental and physiological processes, complications can arise because of different plant hormones influencing the level and sensitivity of other hormones. Such as is seen in secondary effects of other hormones on a mutant deficient in only one hormone, possibly because of common steps in the signal transduction pathways of the different hormones, known as cross-talk (Wilson *et al.*, 1990; Bitoun *et al.*, 1990). Transgenic technologies can allow the additive, synergistic or negative effects of a number of different hormones to be investigated. For example, the *iaaM* transgene, and the *ACCase* transgene were used to see the phenotypic effects of increased auxin levels in the absence of ethylene, who's synthesis is stimulated by auxin (Romano *et al.*, 1993). The *iaaM* gene increases free indole-3-acetic acid (IAA) concentrations whilst the *ACCase* gene destroys ACC, the rate-limiting substrate in the ethylene biosynthetic pathway.

Three transgenes that have been used to manipulate auxin levels are the *iaaL* gene from *Pseudomonas syringae*, and the *iaaM* and *iaaH* genes from *Agrobacterium tumefaciens*. The *iaaL* gene product is a IAA-lysine synthetase, which converts IAA to IAA-lysine, a conjugate that has considerably reduced auxin activity (Glass and Kosuge, 1986; Roberto *et al.*, 1990). When it is expressed in a transgenic plant, such as potato (Spena *et al.*, 1991), tobacco (Spena *et al.*, 1991; Romano, Hein and Klee, 1991), or *Arabidopsis* (Estelle and Klee, 1994), conjugation is increased, so removing IAA from the active pool of auxin and effectively reducing auxin levels within the plant. The *iaaM*

gene product converts tryptophan to indoleacetamide (IAM), whilst the *iaaH* gene product converts IAM to IAA. So, when these two transgenes are expressed together in the same plant the endogenous concentration of IAA increases.

Because of the lack of available cytokinin overproducing mutants, the best approach to investigating the role of cytokinins is through the over expression of the *ipt* gene form the T-DNA of *Agrobacterium tumefaciens*. The gene encodes an *iso*pentenyltransferase catalysing the formation of *iso*pentenyladenosine-5-phosphate from adenosine-5-phosphate and isopentenyl diphosphate (Barry *et al.*, 1984). Plants are able to convert this intermediate into active cytokinins. Similarly with auxin, cytokinin degradative enzymes can be used to reduce cytokinin levels within a particular tissue.

The major consideration when using these transgenes is where and when to activate them within the plant. The use of specific promoters to drive the expression of genes is just as crucial to the integrity of endogenous manipulation as a technique, as simply increasing or decreasing hormone levels under a constitutive promoter is essentially how exogenous application works. The only limiting factor to the success of this technology is the availability of promoters that can give the required specific tissue and developmental expression of the transgenes. An alternative is to use inducible promoters that can ensure the expression of the transgene under specific conditions, such as dexamethasone inducible or heat-shock inducible promoters.

1.2.6 Conclusions

The advancement in our understanding of how plant hormones work has been due to the advancement of molecular genetic techniques, and this will surely continue to be the case in the near future. We now have models of perception and signal transduction leading to changes in gene expression, upon which future experiments can be based. However, this knowledge is more meaningful once it is applied in a developmental context, where the role and interplay of each of the major hormones can be seen in space and time. With this in mind the following two sections will look at the importance of signalling events within embryogenesis and post-embryonic development, focusing on the role of hormones and other less well known signalling molecules in co-ordinating and orchestrating patterning events.

Part Three: Signalling in *Arabidopsis* Embryogenesis

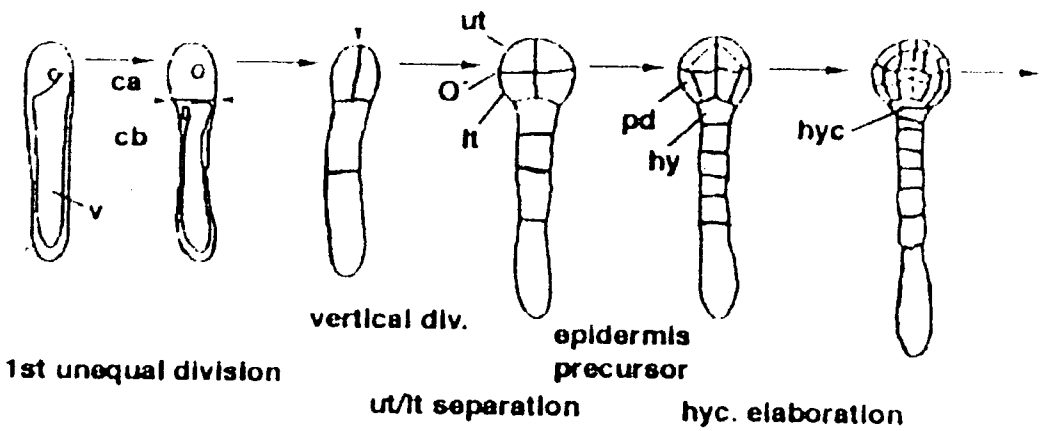
1.3 *Arabidopsis* Embryogenesis

Embryogenesis offers an excellent system in which to see pattern formation and the signalling mechanisms that underpin its establishment and development through to the mature seedling. By looking at how the plant, from a single cell, develops the complex pattern of the mature embryo, we can gain useful insights into how post embryonic processes continue the elaboration of this pattern in response to the external environment. Indeed, our understanding of signalling in seedling development is also helping to expand our ideas about signalling in embryogenesis.

Embryogenesis in *Arabidopsis* is a highly predictable, regulated and organised process. In this section we will look at each stage and consider some of the signalling mechanisms that have been shown to be involved in directing embryogenesis, as well as discuss other signal molecules that may have a role to play.

The two diagrams below show the development of the zygote, from the first unequal division through to the mature embryo.

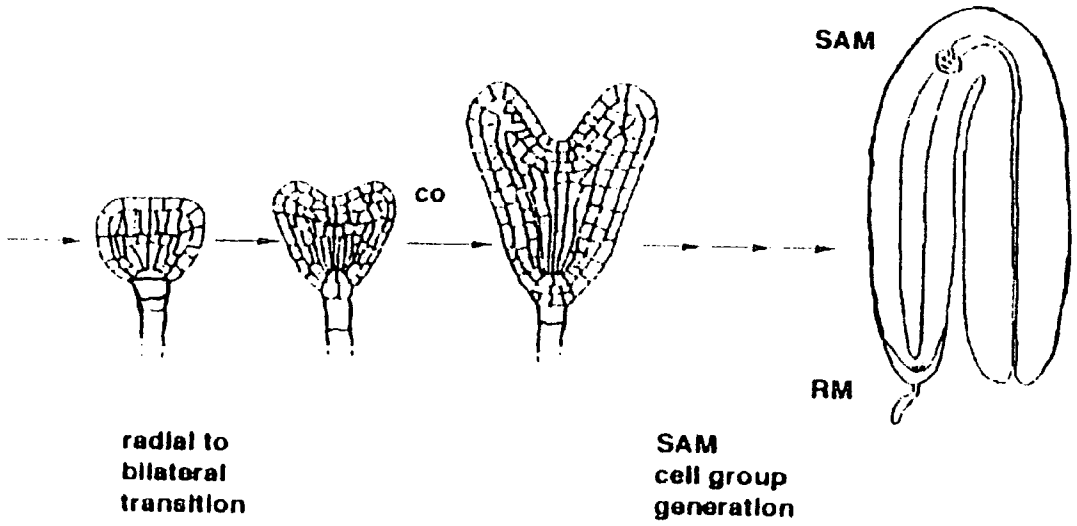
Figure 1.3 *Arabidopsis* Embryogenesis (Part One)



Key:

apical cell (ca)	vacuole (v)	upper tier (ut)
basal cell (cb)	O' boundary (O')	lower tier (lt)
protoderm (pd)	cotyledon (c)	hypophyseal cell (hy)
hypophyseal cell group (hyc)		

Figure 1.3 *Arabidopsis* Embryogenesis (Part Two)



(Figure taken from Torres Ruiz, 1998).

From the first unequal division of the zygote there is a crucial need for signalling in order to ensure that cell division planes are correctly oriented, which leads to the need for positional information and co-ordinating signals.

1.3.1 Cell Fate Decisions: Embryo-Proper versus Suspensor

The observed apical-basal polarity in the zygote of *Arabidopsis* presages polar development during embryogenesis. The zygote undergoes an asymmetric transverse division to generate two daughter cells that are of unequal size and follow distinct developmental pathways. The basal cell (Figure 1.03.01a, cb), which is the larger of the two, derives from the vacuolar region of the zygote (Figure 1.03.01a, v) , while the smaller upper cell derives from the cytoplasmic region (Figure 1.03.01a, ca). The upper cell then divides to form the embryo proper, while the basal cell forms a single file of typically six to nine cells, the suspensor. Only the uppermost cell of the suspensor, the hypophysis (Figure 1.03.01a, hy), contributes to the embryo proper as part of the root meristem (Dolan *et al.*, 1993; Scheres *et al.*, 1994). The suspensor appears to have a number of different functions: it physically projects the embryo into the endosperm, and provides both a conduit and a source of hormones and nutrients for the developing embryo. Perhaps the most clear difference in fate between the embryo-proper and

suspensor is seen as the programmed cell death of the suspensor when the embryo reaches the torpedo-stage of development (Yeung and Meinke, 1993).

There is also increasing evidence that the embryo and suspensor express distinct gene expression programmes. While a number of embryonic mutations, such as *knolle*, *fass*, *gnom/emb30* and *hobbit* (Lukowitz *et al.*, 1996; Torres Ruiz and Jurgens, 1994; Mayer *et al.*, 1993; Willemsen *et al.*, 1998) affect the cellular organisation and/or division activity of the embryo, hypophysis and suspensor, other mutants, such as *hydra1*, show embryo-specific defects (Topping *et al.*, 1997), suggesting that the *HYDRA1* gene is expressed in the embryo but not in the suspensor. Direct evidence for different gene expression profiles in embryo and suspensor comes from promoter trap analysis in *Arabidopsis*, which has led to the identification of genes that are specific to the embryo-proper (Topping *et al.*, 1994; Topping and Lindsey, 1997) and to the suspensor (P. Gallois, University of Manchester, unpublished). Differences in gene expression between the apical and basal cell following the first zygotic division have also been identified. For example, the apical cell has been shown to accumulate the *ARABIDOPSIS THALIANA MERISTEM LAYER 1* (*AtML1*) gene transcript, which is not detected in the basal cell (Lu *et al.*, 1996).

A Role for Cell Wall Components?

The establishment of two separate cell fates for the upper and lower cell must involve a signal of some kind, either from the surrounding maternal tissue or from within the zygote itself. However, because of the fact that there are only two cells, there is a limit to how complex this signal system can be, so a good starting point was to look for differences between the two cells, and at the time this was purely a physical search as the genetic tools available at the time were not sensitive enough. It is only now that the genetic consequences of these differences are stating to be looked at with any degree of certainty.

In pollen development, the formation of the structurally and functionally distinct vegetative and generative cells, and the expression of genes within those cells, has been shown by in vitro techniques to depend on the asymmetry of the formative cell division, pollen mitosis I (Eady *et al.*, 1995). More recently, van den Berg *et al.*, (1995, 1997) have used laser ablation techniques to demonstrate the role of short-range signalling between cells in the *Arabidopsis* root to direct their fates.

A large amount of work has focused this search for physical differences on the cell wall. Much of the evidence for cell wall differences that are cell type- or tissue-specific comes from work in which monoclonal antibodies have been raised in response to immunizations with complex mixtures of plant cell material. By labelling these antibodies and localizing their binding sites in plants, a series of probes has been generated that each recognise cell surface polysaccharide epitopes associated with particular cell types (Knox *et al.*, 1991; Pennell *et al.*, 1991; Pennell *et al.*, 1995). The antibodies recognise components of the pectin matrix of the wall, specifically arabinogalactan moieties attached to proteins in the plasma membrane, the so-called arabinogalactan proteins (AGPs). Interestingly, there are differences in AGP localization during Brassica embryogenesis. For example, the JIM8 antibody reveals cell differences between embryo-proper and suspensor, binding only to the cells whose future fate is as the suspensor (Pennell *et al.*, 1991).

Not only have AGPs been identified that are differentially expressed during zygotic embryogenesis, but they are also differentially expressed during somatic embryogenesis. Somatic embryos develop, not from fertilized egg cells, but from somatic (non-reproductive) cells that have been tissue-cultured. These cells are induced to become structurally disorganised, and lose the characteristics of the differentiated state of the tissue from which they derive. However, they can reorganise if given appropriate hormonal signals (usually a removal of auxin from the culture medium). Despite the fact that they are not in contact with the maternal influences of the seed, they are able to develop in a polar way and to generate embryoidal structures that are similar to zygotic embryos, and indeed can go on to 'germinate' into plants.

The classical system to study somatic embryogenesis is in cultured cells of carrot. In this system, meristematic, relatively undifferentiated cells are grown in liquid medium in the presence of auxin, as globular cell clusters: these have been designated proembryonic masses (PEMs). These probably represent preglobular-stage embryos, arrested in their further development by the presence of auxin. But when transferred to an auxin-free medium, cells of the PEMs become organised to form adventitious embryos (Krikorian and Smith, 1992). It is also possible to induce single cells of carrot to form embryos directly by manipulating auxin - cytokinin concentrations in the culture medium (Nomura and Komamine, 1985; Pennell *et al.*, 1995).

In relation to the question of a role for AGPs in cell fate determination, the single cell embryogenic system is of interest. The single cells divide, and the products of the division have separate fates: one cell becomes an embryonic initial, which undergoes further divisions to form an embryo; while the other cell fails to divide further. The original single cell expresses one particular AGP epitope, recognised by JIM8: and this is indicative of a cell with embryonic potential, shown by video tracking (McCabe *et al.*, 1997). When this cell divides, the cell that becomes the 'embryo initial' switches *off* the JIM8 epitope, while the second cell (the 'nurse cell') continues to express that protein. This is reminiscent of the suspensor cell expression pattern of JIM8 in the zygotic embryo (Pennell *et al.*, 1991), and the two division products of the single cell are analogous to the zygotic apical and basal cell.

But is there evidence that the JIM8 target actually regulates cell fate rather than being a marker of a change in cell fate? To investigate this, McCabe *et al.*,(1997) purified JIM8-positive or JIM8-negative cells, and collected cell wall components released from the walls of each. JIM8-negative cell wall components, lacking that epitope, were found not to continue to divide and form embryos. However, if the JIM8 epitope, collected from the 'nurse' cells is added to the 'initial' cells, they will go on to form embryos; however they require JIM8-positive cell- conditioned medium in order to do so.

This indicates that the JIM8 epitope can be used to identify cells which have a role in cell-cell communication and early cell fate specification in carrot somatic embryogenesis. Indeed, the JIM8 epitope may itself be involved in early events of determination of cell fate in carrot somatic embryogenesis, and also in maintaining activity of division of the embryo: i.e. it may signal to the initial cells to keep dividing. Further support for an inductive effect of AGPs in somatic embryogenesis comes from work described by Kreuger and van Holst (1993, 1995). These authors found that the addition of AGPs from an embryogenic carrot cell line to a non-embryogenic line caused an induction of embryogenic capacity of those cells.

A functional role for AGPs is further supported by Willats and Knox (1996). By treating seedlings of *Arabidopsis* with the β-glucosyl Yariv reagent (Yariv *et al.*, 1962), which specifically binds and cross-links AGPs, they observed a reduced overall growth of shoot and roots. In roots, this correlated with a reduced longitudinal cell expansion and increased radial expansion. Although the precise effect of cross-linking of cell

surface AGPs is unknown, it suggests that their biological activity has been inhibited, activity, which may include a role in control of cell expansion and organogenesis.

Two mutants have been identified that have decreased AGP contents, *diminuto* (*dim*) (Takahashi *et al.*, 1995) and *root epidermal bulger* (*reb1-1*) (Baskin *et al.*, 1992; Ding and Zhu, 1997). *dim* mutants are BR biosynthesis mutants that are rescued by application of BRs (Klahre *et al.*, 1998), so the reduction may be a secondary effect; the mutants suffer from reduced cell expansion. The *reb1-1* mutation can be mimicked by growing plants in the presence of Yariv reagent (Ding and Zhu, 1997). However, whether *reb1-1* is defective in synthesis of a particular AGP or in their processing is not known.

The finding of glycosylphosphatidylinositol (GPI) anchors on AGPs (Youl *et al.*, 1998; Oxley and Bacic, 1999; Sherrier *et al.*, 1999; Svetek *et al.*, 1999a) suggests a signalling role for these proteins. GPI anchors help to anchor the protein to the cell surface in the same manner as a transmembrane domain, and can lead to increased lateral mobility in the membrane, or to polarized transport to the apical surface of cells (Hooper, 1997). A number of animal GPI-anchored proteins have been implicated in signal transduction pathways (Peles *et al.*, 1997; Kleeff *et al.*, 1998; Resta *et al.*, 1998). In these cases, signal transduction occurs through interactions with other membrane-bound proteins, both in the same cell or in neighbouring cells (Peles *et al.*, 1997). An alternate mechanism of action is through cleavage of the protein from its lipid anchor by a phospholipase (Udenfriend and Kodukula, 1995), which could generate both intra- and extracellular proteoglycan components. Structural characterisation of the remnants of the GPI anchor present on *PcAGP1* purified from the culture medium of suspensor-cultured cells of pear suggests that the membrane-bound form is released by the action of a phopholipase (Oxley and Bacic, 1999).

Schultz and colleagues (2000), have used the *Arabidopsis* EST database and a reverse genetic approach to identify a family of 15 genes encoding the protein backbone of classical AGPs. The family shows expression (through mRNA analysis) in different tissues, and a large amount of overlapping expression, which must be expected with such a large gene family. Interestingly they have also identified cleavage sites within the AGP genes, suggesting that *Arabidopsis* has a large family of GPI-anchored AGPs. immunolocalisation and SDS-PAGE analysis showed that some *Arabidopsis* AGPs are

localised to the plasma membrane (Dolan and Roberts, 1995; Dolan *et al.*, 1995; Sherrier *et al.*, 1999). If the *Arabidopsis* AG peptides are released from the cell surface and they are similar in size to the wheat AG peptide (molecular mass ~20 kD; Fincher *et al.*, 1974), then they have the potential to act as signals that can diffuse easily through plant cell walls. Carpita *et al.*, (1979), showed that living plant cells have pores that can allow globular molecules as large as 40kD to pass through. Schultz and colleagues now plan to identify mutations in all of these genes to try and understand the role of this gene family in development.

Yet further evidence for the importance of cell wall components in development also comes from work with the carrot somatic embryogenesis system. One mutant cell line, *ts11*, has been identified that fails to undergo embryogenesis when grown at an elevated temperature, even under conditions which are inductive for non-mutant lines (i.e. auxin-free). At elevated temperatures (32°C), *ts11* embryos arrest at the globular stage. However, it was found that developmental arrest at elevated temperatures could be by-passed by the addition of culture medium in which fully embryogenic lines had been grown. The secreted molecule was identified as a 32kDa protein with homology to an endochitinase (de Jong *et al.*, 1992). In search of a substrate for this enzyme, a range of molecules containing N-acetylglucosamine moieties were added to *ts11* cells, to find compounds which also rescue the mutant, and so might represent natural substrates or products of the chitinase. Interestingly, it was found that the mutant could be rescued by the application of lipo-oligosaccharides to the culture (de Jong *et al.*, 1993). This class of molecule consists of an oligosaccharide backbone of 4 or 5 β-1,4-linked N-acetyl-glucosamine residues with a C16 or C18 fatty acid group attached to the non-reducing end. They are known to act as important signals in the nodulation process following Rhizobium interaction with legume roots, and have been designated Nod factors (Schultze and Kondorosi, 1996). Also, recently presented work by Martine Devic showed that the *Arabidopsis cyclops* mutant undergoes the first division and then stops. The *CYCLOPS* gene encodes an enzyme that makes β-1,4-N-acetyl-glucosamine (M. Devic, personal communication).

Purified Nod factors have a wide range of effects on the roots of legumes: some effects are very rapid, some over a period of days or weeks. The most rapid response is transient depolarisation of the plasma membrane, occurring within 15 seconds. This leads to an increase in intracellular pH, and a spiked oscillation in intracellular calcium

levels (reviewed by Schultze and Kondorosi, 1996). This may represent an activation of an intracellular signal transduction pathway, but a causal relationship has not yet been demonstrated. Synthetic Nod factors can also induce division in tobacco protoplasts, in the absence of auxins and cytokinins, and the fatty acid structure has been shown to be important in this activity (Röhrig *et al.*, 1995). So a common role for lipooligosaccharides in somatic embryogenesis and root nodule formation may be as stimulators of cell division, and at concentrations as low as 10^{-15} M.

One speculative view of the molecular mechanisms of targeted secretion of wall components, and subsequent role in higher plant embryogenesis, derives from the observation that the *GNOM* (*GN*) protein of *Arabidopsis*, which plays a role in Golgi vesicle transport/trafficking protein, and is susceptible to brefeldin A inhibition (Grebe *et al.*, 1998, Steinmann *et al.*, 1999). Brefeldin A can inhibit targeted wall secretion and polar axis fixation in *Fucus* (Shaw and Quatrano, 1996), and, similarly, *gn* mutants, defective in *GN* protein function, are also defective in establishing the asymmetry of the first zygotic division and subsequent apical-basal patterning. Golgi vesicle transport proteins such as Sec7 of yeast, which has similarities to *GN*, have roles in cell wall elongation and in cell division, delivering important precursors for the plasma membrane and the cell wall (Shevell *et al.*, 1994), as well as other proteins that require directional delivery to the cell membrane or wall. *PIN1* localisation in the *gnom* background is severely affected (Steinmann *et al.*, 1999), however the polar localisation of *PIN1* does not occur until the globular stage, which is much later than the observed defects in the *gn* mutants. Such processes require directed and precise delivery of the vesicle. The work of Pennell *et al.*, (1991) demonstrates the differential distribution of the JIM8 epitope along the apical-basal axis of the Brassica embryo-suspensor complex, and the results of McCabe et al (1997) show similarly its targeted and polar distribution in the bicellular embryo-nurse cell complex in the carrot system. It is therefore possible that cell wall components such as the JIM8 epitope are crucial for imparting positional information at the earliest stages of apical-basal axis formation, and require *GNOM* protein function for their correct spatial distribution.

emb30 (allelic to *gn*) mutants, showed normal expression of JIM5 and JIM7 epitopes, however they showed abnormal distribution of other pectins an cell wall materials (Shevell *et al.*, 2000). Pectin forms the major part of the middle lamella and is required

for cell adhesion, and consistent with the abnormal distribution, the *emb30* mutants showed reduced cell adhesion in friability tests. The normal localisation of xyloglucan and members of the AGP family therefore suggests that cell adhesion is required for the interpretation of local signals. However the possibility that other specific AGPs, such as the JIM8 epitope, are delivered by the *GNOM/EM*B30 secretory pathway has not been discounted.

These results therefore suggest a role for cell wall-related molecules in regulating important aspects of embryogenesis and polarity. Whether fertilization induces targeted secretion of wall-localised regulatory molecules in higher plants is still unknown, but is an intriguing possibility.

Genetic Control of Embryo-Suspensor Cell Fate Determination

The fates of the apical and basal cells, following zygotic division in *Arabidopsis*, are clearly distinct. Direct evidence for a genetic control of suspensor cell identity derives from studies of mutants in which the suspensor undergoes abnormal patterns of cell division, most commonly ectopic division. In the abnormal *sus*pensor (Schwartz *et al.*, 1994) and *raspberry* (Yadegari *et al.*, 1994) mutants of *Arabidopsis*, the embryo proper arrests and the suspensor subsequently enters into a series of inappropriate divisions. Significantly, the modified suspensor takes on a variety of characteristics of the embryo-proper. Ultrastructural analysis has revealed that, in the case of the *sus* mutants, for example, accumulation of storage protein bodies, lipid bodies and starch grains occurs in both the embryo-proper and, unusually, the suspensor (Schwartz *et al.*, 1994). It has also been observed that *AtLTP*, which encodes an *Arabidopsis* homologue of the carrot *EP2* lipid transfer protein (Sterk *et al.*, 1991; Thoma *et al.*, 1994), is strongly expressed in the protoderm/epidermis of embryos and seedlings but is not expressed in the wild type suspensor. However, it is expressed in the peripheral cells of the *raspberry* embryo proper and suspensor (Yadegari *et al.*, 1994). Even more spectacular is the re-differentiation of suspensor cells in the *twin* (*twn*) mutants. Here, the suspensor cells re-organise into secondary embryos, following arrest of the embryo-proper (Vernon and Meinke, 1994). The *TWN2* gene has now been cloned, and encodes a valyl-tRNA-synthase, though its mode of action remains unclear (Zhang and Sommerville, 1997).

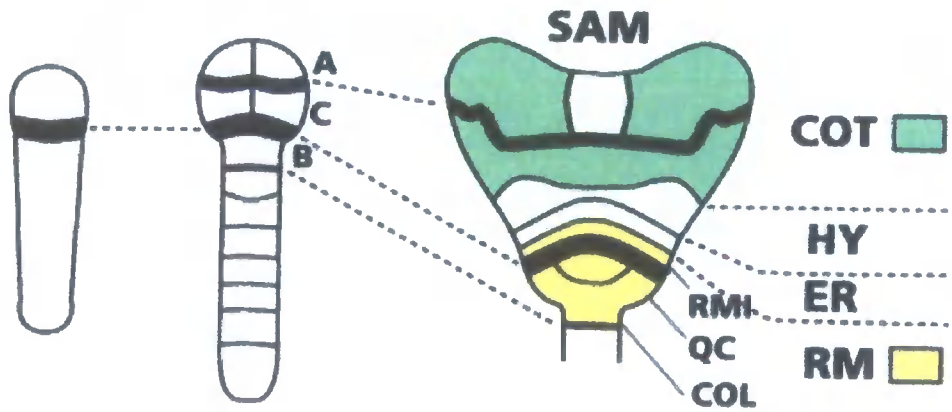
It has been suggested that the wild-type embryo-proper signals to the suspensor to maintain its differentiated state, and in the case of the *sus* and *raspberry* mutants, this

embryonic development (Schwartz *et al.*, 1994). A novel suspensor mutant of *Arabidopsis* has been identified, designated *asf1* (for *altered suspensor fate1*), that exhibits a novel pattern of inappropriate cell division in the suspensor, and a reprogramming of gene expression and cell differentiation. Activation of auxin-inducible genes in the modified suspensor suggests that the mutant phenotype is mediated by the de-regulated partitioning of auxin between the embryo-proper and the suspensor, to activate the observed ectopic cell division (Horne, 1998). The recently described *axr6* mutant, which is auxin resistant and shows supernumerary suspensor cells (Hobbie *et al.*, 2000), also lends support to this model. We will return to the likely role of auxin in the patterning of the basal region of the embryo later.

1.3.2 Apical-Basal Patterning: The Embryo-Proper and Seedling

The apical-basal pattern is defined by the positioning of the shoot meristem and cotyledons, the hypocotyl and the root and root meristem. The study of mutants has led to the theory that the embryonic axis is therefore partitioned into three main areas or clonal regions; apical (A), central (C) and basal (B) (Mayer *et al.*, 1991),

Figure 1.3.2 Clonal Regions of the Embryo



(Figure taken from http://www.bio.uu.nl /~mcbroots/fatemap.htm)

The shoot meristem (SAM) and the majority of the cotyledons (COT) originate in the apical region, while the central region contributes to the majority of the rest of the axis, namely the shoulder of the cotyledons, the hypocotyl (HY), the embryonic root (ER), and the root meristem initials (RMI). It is only the quiescent centre (QC), the columella

and the root meristem initials (RMI). It is only the quiescent centre (QC), the columella initials and the central root cap (COL) that arise from the clonally separate hypophyseal cell, the upper-most suspensor cell, whilst the rest of the pattern is derived from the embryo proper (Scheres *et al.*, 1994; Mayer and Jürgens, 1998). Despite the temptation to consider the formation of each of the three regions as independently regulated events, it will become clear that interactions between tissues in each region are essential for the correct integrated patterning of the whole seedling. For convenience, however, we will examine relevant features of each of the three regions respectively.

Each region follows its own programme of cell divisions once they have been established, all three being present by the octant stage. The formation of the O' boundary at the quadrant stage creates the upper and lower tiers, corresponding to the apical and central regions respectively, whilst the hypophyseal cell is formed by divisions in the suspensor. The apical region divides without preferential orientation, while divisions that are perpendicular to the axis create the cell files that characterise the central region. Within the basal region a more stereotyped set of divisions is required to create the root meristem and central root cap, such that the fate of any cell in that region can be predicted with high probability (Scheres *et al.*, 1994).

Through studying the development of each of these regions in both wild-type and mutant backgrounds, the different signalling mechanisms involved are becoming clearer. Much progress has come from the application of a strategy of mutagenesis and the progressive isolation and characterisation of genes that are specifically involved in embryonic pattern formation. It is worthwhile to note that as the embryonic pattern is reiterated through the meristems during postembryonic development, many defects that originate in the embryo are often identifiable in seedling mutant screens.

What then are the mechanisms that generate positional information to promote region-specific gene expression patterns? We will now look at the genes that specify cell fate within the *Arabidopsis* apical-basal axis, and consider evidence for the signalling events involved.

1.3.3 The Apical Region of the Embryo

The apical region forms the self-perpetuating shoot meristem and the majority of the cotyledons. The specification of the shoot meristem starts when the four inner apical cells at the 16-cell stage *Arabidopsis* embryo start to express the *WUSCHEL* (*WUS*) gene, which is an early marker of the shoot meristem cell fate (Mayer *et al.*, 1998). *WUS* is expressed through a number of asymmetric divisions that also produce the future cotyledonary primordia, however *WUS* expression is not required for these cells to follow this fate. As a consequence of these asymmetric divisions, expression is restricted at the globular stage to the group of cells at the apex of the embryo that will become the shoot meristem primordium (Laux *et al.*, 1996). The *WUS* gene has been shown to encode a novel homeodomain protein (Mayer *et al.*, 1998). A possible role for *WUS* at this early stage in development is to maintain the pluripotent capacity of the shoot meristem precursor cells (Lenhard and Laux, 1999).

The *GURKE* gene of *Arabidopsis* is also required for the correct organisation of the shoot apical region (Torres Ruiz *et al.*, 1996). Strong mutant alleles are unable to construct the entire apical region, and even part of the hypocotyl, while weaker alleles produce abnormally shaped leaves and flowers. The root and radial patterning is apparently unaffected, even in strong mutant alleles. The defect can be traced back to the transition-stage embryo.

SHOOT MERISTEMLESS (STM) expression is initiated at the late globular stage in the central region of the embryo apex (Long *et al.*, 1996), and is independent of *WUS* action, of which it is a positive regulator (Mayer *et al.*, 1998). *stm* mutants have fused organs originating from the shoot meristem, which suggests that *STM* is required to prevent the incorporation of the central meristem cells into organ primordia (Long *et al.*, 1996; Long and Barton, 1998; Endrizzi *et al.*, 1996). *STM* is expressed by only a specific set of cells within the apex of the embryo, and has been shown to encode a member of the *KNOTTED* homeodomain proteins. *AINTEGUMENTA (ANT)*, meanwhile, is expressed by the two cell groups which flank the shoot meristem, and which eventually form the cotyledons (Elliott *et al.*, 1996).

STM is expressed throughout the seedling meristem and so may be a more general regulator of meristem organisation, preventing meristem cells from being recruited into organs. *WUS* has to be switched off for organ initiation.

In the heart stage embryo, when cotyledon primordia emerge, *CLAVATA1 (CLV1)* expression is initiated in the stem cell population in the shoot apex. *CLV1* encodes a membrane-bound receptor protein kinase (Clark *et al.*, 1996), and acts independently of *STM* (Long and Barton, 1998). The *CLV* genes promote the progression of meristem cells towards organ initiation. *POLTERGEIST* is a possible downstream target of *CLV3* (Yu *et al.*, 2000).

It has emerged that the *CLAVATA* signalling pathway has an important role in negatively regulating the accumulation of stem cells within the seedling shoot meristem (Brand *et al.*, 2000; Schoof *et al.*, 2000). *CLV1* and *CLV3*, receptor and ligand respectively, interact at the plasma membrane surface (Brand *et al.*, 2000). Subjacent to the *CLV1* and *CLV3* expressing stem cells is an 'organising' centre, which expresses *WUS*. The *WUS* expressing 'organising' cells stimulate expression of *CLV3* in the overlying stem cell population. *CLV3* binds to *CLV1*, which then represses the expression of *WUS* at the transcript level, forming a feedback loop that regulates the size of the stem cell population (Schoof *et al.*, 2000).

One gene that does interact with *STM* is *ZWILLE* (*ZLL*, Moussian *et al.*, 1998). The *zll* mutant shoot meristem is initiated correctly, but *STM* expression is either restricted or down regulated, resulting in cells which follow other development fates, possibly due to the influence of other spatial cues. *ZLL* is therefore required to maintain meristem cell identity within the apex, possibly through acting as a translational control. *ZLL* codes for a member of a novel gene family of translation factors. The *ZLL* gene is expressed in the vascular precursor cells, situated just below the meristem primordia, from early stages until leaf primordia are established, when presumably the meristem can maintain itself.

The analysis of these genes has shown that position-dependent cell fate specification is achieved from the late globular stage onwards. It appears that meristem formation occurs through the activation of genes that specify cell fate in a spatially precise manner. A key area of research has been to identify possible signals that may activate

and regulate the expression of the genes described above. Both cytokinin and auxin have been implicated as positional signals within the apical region of the embryo.

The *altered meristem program1* (*amp1*) mutant has both larger meristems as well as higher levels of cytokinin (Chaudhrey *et al.*, 1993; Chin-Atkins *et al.*, 1996). Work by Rupp *et al.*, (1999), has confirmed this observation of a larger shoot meristem in *ipt* expressing transgenic *Arabidopsis* and Tobacco plants. They found that *KNAT1* and *STM* mRNA was up-regulated in these lines, and that *KNAT1* over-expressing lines resembled cytokinin overproducing plants.

Cytokinins are known to induce the formation of organised shoot meristems from disorganised callus (Skoog and Miller, 1957), and the basic signal-generating system for vegetative programming of the meristem is believed to be located within the meristem itself (Sussex, 1989). Both immunological and direct measurement data show high levels of cytokinin in mitotically active areas such as the shoot and root meristem (Mok and Mok, 1994; Dewitte *et al.*, 1999). Cytokinin may therefore be the cause of the increased meristem in *amp1* mutants, or the increased meristem may have a knock-on effect upon the synthesis of cytokinin.

AMP1 has now been cloned and shown to encode a 706 amino acid glutamate carboxypeptidase enzyme (Helliwell *et al.*, 2001), that is similar to N-acetyl α-linked acidic dipeptidases that cleave signalling peptides in eukaryotic systems. At present there is no obvious candidate peptide that is processed by the *AMP1* gene product. The biological role of *AMP1*s substrate is therefore one of two possibilities. Either its primary effect is upon meristem size, which indirectly increases cytokinin biosynthesis, or it directly increases cytokinin biosynthesis which then increases the size of the meristem as a result. Initial observations are insufficient to determine between these two possibilities.

A second mutant with high levels of cytokinin has recently been characterised. The *high shoot organogenic capacity* (*hoc*) mutant has a 2-fold higher level of cytokinin in the shoot and a 7-fold increase in the root (Catterou *et al.*, 2002). There is also a small increase in the level of auxin when compared with wild type, and mutants are able to regenerate without the supply of exogenous auxin or cytokinin. *amp1* mutants have extra leaves within a normal phyllotaxis, whilst the *hoc* mutants have supernumerary

rosettes and precocious axillary meristem development, although they also maintain the a correct phyllotaxis. *hoc ampl* double mutants have numerous leaves and rosettes. Although the *HOC* gene has not been cloned, the mutant phenotype suggests that there is a role for cytokinin in the organisation of the shoot region.

Cytokinin may therefore provide positional information for the meristem and thus aid its regional organisation, or it may simply regulate cell division within the stem cells and initials. Cytokinin is known to induce cyclin gene expression (Hemerly *et al.*, 1993; Soni *et al.*, 1995) and to cycle cells through the cell cycle. Cytokinin may therefore act upstream of homeobox genes that regulate the size of the shoot meristem.

Auxin Acts as a Positional Signal for the Outgrowth of the Cotyledons and the Transition to Bilateral Symmetry

Auxin has been proposed as a key signal molecule in providing positional information within the apical region of the embryo, particularly during the transition period from globular to heart stage. Liu *et al.*, (1993) first reported the use of auxin transport inhibitors to study development in cultured zygotic embryos of *Brassica juncea*. They showed that inhibition of auxin transport at the globular stage leads to the formation of embryos that lack bilateral symmetry at the heart stage. Bilateral symmetry is established when the two cotyledons form either side of the shoot meristem region. Instead of two cotyledons, embryos developed with fused and collar-like cotyledons, which interestingly phenocopied known auxin transport defective mutants *pin1* (Okada *et al.*, 1991) and *gnom* (Steinmann *et al.*, 1999).

Hadfi *et al.*,(1998) used this same *B. juncea* culture system to look at the effects of auxin (IAA), an anti-auxin (PCIB), and an auxin transport inhibitor (NPA). When auxin was supplied, ball-shaped or cucumber-shaped embryos resulted, possibly because the embryo, flooded with exogenous auxin, is unable to establish the auxin gradients that are essential for morphogenesis. The anti-auxin PCIB inhibited cotyledon growth so that either only one or no cotyledons developed. Correct hypocotyl and radicle growth was also found to require auxin action and movement. Furthermore, when globular-stage embryos were treated with exogenous NPA, axis duplication was seen, whilst a later application produced split-collar or collar-like cotyledons. These results confirm the findings of Liu *et al.*,(1993), and help clarify the model of auxin movement which they first proposed: continuous auxin transport removes auxin from the area between

the two emerging cotyledons, and supplies the auxin back to the cotyledonary primordia. Auxin removal starts in the central apical region of the globular or early transition embryo, and continues asymmetrically across the apex of the embryo.

Inhibition of auxin transport therefore blurs the positional information that is created by its normally precise redistribution, resulting in increased cell division throughout the shoot apex. These findings indicate that auxin translocation is a prerequisite for the radial globular embryo to progress to the bilaterally symmetrical heart stage embryo. Similar results were found by Fischer *et al.*, (1997) for morphogenesis of the embryo of the monocot wheat, suggesting that there are overlapping mechanisms at work here.

During *Arabidopsis* embryogenesis, *PIN1*, which is localised at the basal end of cells within the vascular stele (Galweiler et al., 1998), becomes polarised in its expression pattern at the mid-globular stage, before the two cotyledons have started to develop. By the heart stage the pattern very much resembles the pattern it takes throughout the rest of the plant's postembryonic development, forming a characteristic Y shape from the two cotyledons to the basal end of the embryo (Steinmann *et al.*, 1999).

More recent work has taken this model further and clarified the role of directed auxin transport in the position and separation of the two cotyledons seen in *Arabidopsis*. Vernoux *et al.*, (2000), have shown that *pin1* mutant inflorescences have normal meristem organisation, suggesting that meristem patterning does not require *PIN1* or a strict regulation of auxin transport. However the cells at the periphery of the meristem show a mixed identity, expressing *LEAFY COTYLEDON* (*LFY*) and *ANT*, two early markers of organ initiation, as well as *CUP-SHAPED COTYLEDON2* (*CUC2*), a gene required for the separation of the cotyledons (Aida *et al.*, 1999). *PIN1* is therefore required to establish these separate identities, between the primordia and the boundary.

So far two genes in *Arabidopsis* have been clearly associated with the establishment of organ boundaries at the edge of the shoot meristem. *CUC1* and *CUC2* code for two functionally redundant NAC domain proteins, that consequently only show a cup-shaped cotyledon phenotype when mutated in the same plant. At present their biochemical function is unknown. *CUC2* is expressed in the early to mid-globular stage in the presumptive meristem region above the O' line. At the late globular stage expression is seen across the top half of the embryo, but this then becomes restricted to

the areas between the cotyledons, when they emerge during the heart stage. Expression in this area continues throughout the rest of embryogenesis, with expression seen between the SAM and the edge of the cotyledon (Aida *et al.*, 1997; Aida *et al.*, 1999; Ishida *et al.*, 2000).

PIN1 therefore feeds auxin into the organ primordia, which up-regulates *PIN1* expression and switches off *CUC2*, restricting it to the boundary between the meristem and the cotyledon edge. The primordia then inhibit the formation of new primordia in their vicinity, either by producing an inhibitive signal or using up an activator (Lyndon, 1998). *PIN1* is therefore essential for primordia outgrowth and separation, and therefore important in establishing a phyllotactic pattern (Vernoux *et al.*, 2000). The findings of Reinhardt *et al.*, (2000) confirm auxin's role in initiation of organs within the radial plane. They found that application of auxin transport inhibitors to the apex of an inflorescence completely inhibited organ initiation, whilst local application of auxin microdroplets restored organ formation within the peripheral zone. The position of the organ within the peripheral zone is therefore determined by the localisation of auxin, however the identity of the organ is not specified by auxin.

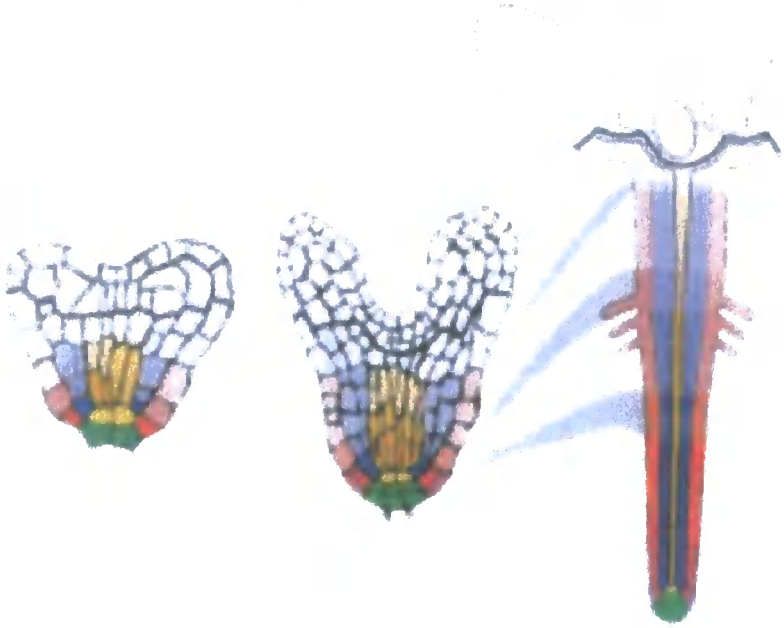
The size of the meristem is therefore of importance for the correct establishment of spatial cues that will be needed by the organ primordia in the embryo as well as postembryonically. A larger meristem could blur the spatial cues and therefore the competence of cells to respond to inductive and repressive signals (auxin or inhibiting signals from primordia to stop other primordia developing in the area).

1.3.4 The Central and Basal Regions of the Embryo

The central part of the embryo produces the majority of the embryonic axis, and a number of mutants have been found that are defective not only in the generation of hypocotyl and root, but also the radial axis within this region. Indeed, the radial organisation of the seedling established during embryogenesis defines the cellular patterning that runs throughout the hypocotyl and the root (Scheres *et al.*, 1995).

The diagram below shows a cell fate map for the central and basal regions of the embryo.

Figure 1.3.4.1 Cell Fate Map for the Central and Basal Regions



(Figure taken from http://www.bio.uu.nl /~mcbroots/fatemap.htm)

The *MONOPTEROS (MP)* gene is required for the formation of the hypocotyl, root, root meristem and root cap; products of the central and basal regions of the embryo (Berleth and Jürgens, 1993), see the figure 1.3.4.2 of two *mp* mutants below. The *MP* gene is also required for correct cell axialization and development of aligned vascular strands (Przemeck *et al.*, 1996). The *MP* gene has been cloned and found to encode a transcription factor with nuclear localisation sequences and a DNA binding domain that is highly similar to a domain which binds auxin-inducible promoters. In fact *MP* has the same binding specificity as *AUXIN RESPONSE FACTOR1* (*ARF1*; Ulmasov *et al.*, 1997a), which is a transcription factor that binds to *ARE*s within promoters of auxin-inducible genes. Expression of *MP* is initially in broad domains in the embryo, becoming eventually confined to the procambial tissues (Hardtke and Berleth, 1998). This is similar to *PIN1* expression, although *PIN1* has been shown not to require *MP* gene function (Steinmann *et al.*, 1999; Palme and Galweiler, 1999). *MP* is therefore required for correct cell axialisation in the early embryo, and for correct vascular development in the later stages of embryogenesis and during postembryogenic development, through its likely role in regulating the transcription of auxin responsive genes.

Figure 1.3.4.2 *monopteros* Mutants

Whether the central region of the *mp* mutant fails to recover from its altered axialisation and therefore cannot recover hypocotyl and root formation, or if the basal region's failure to generate the root meristem is because of a lack of aligned vascular primordia, is not known. There is a large amount of evidence to indicate that auxin is required for root formation (e.g. Boerjan *et al.*, 1995; Celenza *et al.*, 1995; Reed *et al.*, 1998). However, if the central section of the embryo does not develop correctly, then the corollary of this for the basal region must be considered. The *MP* gene is required for correct alignment of the vascular tissue, and cell axialisation within the hypocotyl (Przemeck *et al.*, 1996). It is therefore open to suggestion that the defective polar auxin transport system may causes downstream effects on root development in the *mp* mutant.

Does the central region signal to the basal region to enable the correct development of the latter? There is growing evidence that signalling between embryonic domains establishes the positional information that allows cells to activate fate-determining gene expression programmes.

The *BODENLOS* (*BDL*) gene of *Arabidopsis* has been implicated in auxin-mediated apical-basal patterning processes (Hamann *et al.*, 1999). Development in *bdl* mutants is disrupted at the two-cell stage, when the apical cell divides horizontally rather than vertically. Hypophyseal development is subsequently compromised, leading to mutants that lack an embryonic root (quiescent centre and central root cap). Hence the name, *bodenlos* simply translates as lost-bottom. Hypocotyl development is also affected in some mutant individuals. Interestingly, *bdl* mutants show insensitivity to the synthetic

auxin 2,4-D within the same range as *axr1* seedlings, which suggests that auxin-mediated signalling is required to specify the fate of the basal region of the embryo. Furthermore, the *BDL* gene only affects the embryonic root, since *bdl* seedlings can still form lateral root meristems.

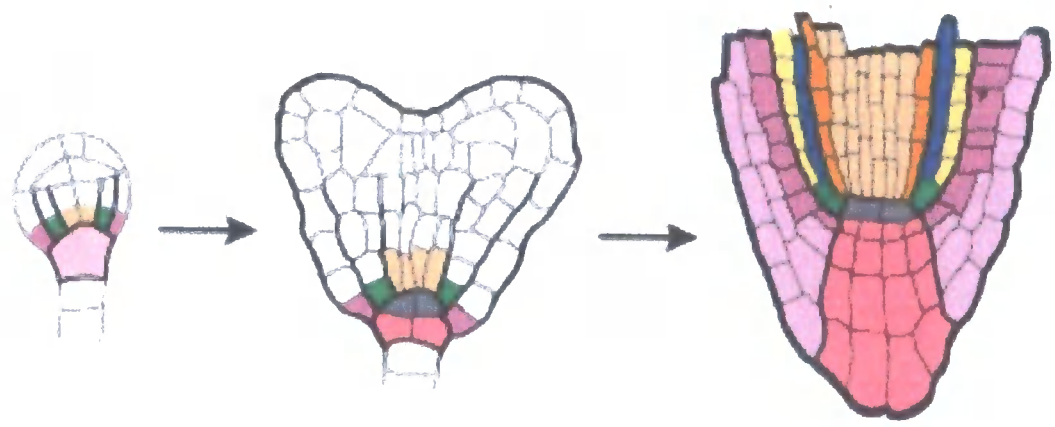
BDL has recently been cloned and shown to be *IAA12*, an *AUX/IAA* gene (T. Hamann, personal communication). As discussed before, AUX/IAAs block the transcription of auxin induced genes by binding to ARFs, which can only dimerize and bind to *ARE*s when they are not bound to AUX/IAAs (Ulmasov *et al.*, 1999). The SCF^{TIR1} complex and the ubiquitin degradation pathway are required to degrade and so remove these transcriptional repressors (Gray *et al.*, 1999). Further more, *MP/ARF5* and *BDL/IAA12* interact in a yeast two-hybrid system, and show an additive/interaction when double mutants are made (T. Guilfoyle, personal communication; T. Hamann, personal communication).

Interestingly *BDL* is only expressed in the embryo proper and not in the hypophysis, where its phenotype is seen. The non-cell autonomous nature of this mutation suggests that a signal is needed from the embryo, at the octant stage, in order to recruit the hypophyseal cell. Later, at the heart stage, the quiescent centre signals to the cells above it to block differentiation, conferring the fate of root meristem initials (Hamann *et al.*, 1999). Studies show that ablation of the quiescent centre in seedlings results in the differentiation of the adjacent initial cells (van den Berg *et al.*, 1997).

auxin resistant6 (AXR6) mutant seedlings are arrested in their development soon after germination, and lack a root and hypocotyl (Hobbie *et al.*, 2000). The stronger *axr6-1* allele has more severe vascular defects than the weaker *axr6-2*, and tends to produce only one cotyledon. Mutants are also more resistant to auxin, undergoing irregularly timed and oriented cell divisions, which are first observed in the early embryo. Principally the suspensor is disrupted by cell divisions that create radial layers rather than the characteristic single file of seven to nine cells. As a result, the hypophyseal cell does not form correctly, and the distinction between the embryo proper and the suspensor is lost. Within the central region the vascular precursor cells fail to establish during the globular stage, a defect which is also seen in *monopteros* (Przemeck *et al.*, 1996). *AXR6* therefore represents a novel gene that causes defects in cell division patterns within the embryo and the suspensor. It is feasible that the aberrant cell

divisions occur because there are problems in auxin-mediated positional or cell-fate signalling. Indeed, the similarities between the phenotypes of the *mp*, *bdl* and *axr6* mutants suggests that they may function in similar pathways (Hobbie *et al.*, 2000). Different ARFs can interact with a number of different AUX/IAAs (Ulmasov *et al.*, 1999; Tiwari *et al.*, 2001).

Figure 1.3.4.3 Fate Map Showing the Embryonic Origin of the Root Meristem



(Figure taken from http://www.bio.uu.nl /~mcbroots/fatemap.htm)

The *HOBBIT (HBT)* gene is required for correct hypophyseal cell formation (Willemsen *et al.*, 1998). *hbt* embryos have incorrect hypophyseal cell development from the quadrant stage onwards, so that by the heart stage activation and formation of the lateral root cap layer has not occurred. Mature embryos lack a quiescent centre and columella root cap. Root meristem formation is not only defective in the embryonic root but also in the seedling, where secondary roots fail to form, even when cultured. *HBT*, unlike *BDL*, is therefore required for root meristem formation both embryonically and postembryonically.

HBT has now been cloned and shown to be a subunit of the Anaphase Promoting Complex (APC) (B. Scheres, personal communication), which promotes cell cycle progression. The APC complex has similarities to the SCF^{TIR1} complex that plays such an important role in auxin signalling, and degrades elements holding the chromatids together as well as cyclins in order to allow progression through the cell cycle. The *HBT* transcript co-localises with *cyclin B* in M phase of mitotically active cells. However,

despite cloning the gene, it is unclear what the exact role of the *HBT* gene is, and how the phenotype is caused by this mutation. Possibly it is required for the correct division programme that the hypophysis must go through to produce the root meristem and root cap, taking its cue from auxin. The phenotype may therefore result from an inability to respond to this auxin signal, which requires proteolytic processing in order to activate the correct genes.

The correct patterning of the root therefore would appear to depend on signalling between the central and basal regions of the embryo, as well as the cell-cell communication that is established once the root meristem becomes active.

Auxin: Important For The Top, But More Important For The Bottom

So it appears that auxin transport down the embryo from the shoot to the root is essential for the correct development of the hypocotyl (axialization) and the recruitment of the hypophysis to form the root meristem. Some very exciting work to investigate this is being done in Remko Offriga's lab, where they are using the *mGAL4-VP16 - UAS* transactivation system to specifically increase or decrease auxin levels within areas of the developing *Arabidopsis* embryo (D. Weijers, R. Offriga & J. Haseloff, personal communication). This molecular system allows for the directed, cell-type specific alteration of auxin levels, or other hormones for that matter. The bacterial *mGAL4-VP16* transactivator is fused to a plant specific promoter, such as *STM*, *DR5* or *LTP1*, and then transformed into a plant. A second construct is then made, fusing the *UAS* promoter, which is specifically activated by the *mGAL4-VP16* protein, to either the bacterial *iaaM* (auxin biosynthetic) or *iaaL* (auxin conjugating) genes. In order to visualise the expression of this construct a GFP-GUS protein fusion is also added to the construct before being transformed into a second plant. The different combinations can then be introduced into the same plant by crossing, with the first generation of embryos being screened for defects.

This system has provided some very interesting results, which confirm in a more precise way then ever before the importance and role of auxin within different regions of the embryo. Embryos can develop normally even in the presence of high *iaaM* activity, suggesting that even at early stages the embryo has a good ability to conjugate or transport auxin. Protodermal elevation of auxin levels can result in embryo asymmetry, but mainly results in embryo arrest.

The most interesting results have been with the *iaaL* construct, with defects mainly in the basal region of the embryo. Depletion of auxin from the entire embryo leads to disruption of the basal, but not the apical region of the embryo. Indeed it seems that depletion of auxin can affect the cotyledon primordia to allow outgrowth, by this is the only apical region defect seen. Depleting auxin levels in the apical region or even the endosperm leads to defect in the basal region.

Auxin is required at early stages to suppress the division of the upper suspensor cell and to allow definition of the root meristem precursors. Use of the *DR5* promoter, which is expressed in the hypophyseal cell group, shows that a physiological peak is required for the proper initiation of this region. Depletion of auxin from the suspensor interferes with hypophyseal cell function, indicating that auxin is required for the maintenance as well as initiation of the promeristem.

The isolation of an *AUXIN BINDING PROTEIN1* (*ABP1*) knockout mutant has reinforced the importance of auxin perception in the development of the early embryo as well as that of the suspensor. *abp1* mutant embryos arrest at the globular stage; cells fail to elongate correctly whilst aberrant cell divisions are seen in both the suspensor and the embryo proper (Chen *et al.*, 2001).

1.3.5 Other Players?

Although much of the discussion so far has focused on the role of auxin, the integration of many different signals will be critical during embryonic development. Cytokinin has well established links with a number of post-embryonic developmental programmes, but has until recently received relatively little attention in embryogenesis, partly due to the lack of cytokinin signalling mutants. This situation is changing rapidly.

The *wooden leg* (*wol*) mutation causes a reduced number of cells within the *Arabidopsis* vascular tissues, all of which differentiate into xylem (Scheres *et al.*, 1995). This phenotype is evident during embryogenesis, when the vascular tissue precursors are laid down. It appears that there is a reduction in layer-specific cell division that causes this reduction in vascular primordia (there are 10 cells inside the pericycle layer compared with 18 in wild type; Scheres *et al.*, 1995). *WOL* has been cloned and found to be allelic to *CYTOKININ RESPONSE1* (*CRE1*), a two-component histidine kinase receptor that is

activated by cytokinin to initiate phosphorelay signalling (Inoue *et al.*, 2001). As mentioned above, cytokinin is known to promote the cycling of cells through the cell cycle, and this may account for the reduced number of divisions within this embryonic tissue. It is also possible that the lack of phloem within this mutant is because all the cells within the vascular tissues respond to auxin before they can respond to cytokinin, and so become xylem vessels instead of phloem.

Vascular tissue formation follows the flow of auxin (Aloni, 1987; Mattsson et al., 1999), which is canalised into files of cells so that connected vascular strands form (Sachs, 1991). Phloem development is stimulated at low auxin to cytokinin ratios, whilst high auxin to cytokinin ratios promote xylem development, and so the density and ratio of xylem to phloem depends on the combination of the auxin and cytokinin signal within the tissue (Aloni and Zimmerman, 1983). This response may also account for the reduced amount of cells within the vascular tissue of the *wol* mutant, as the xylem differentiation process would preclude the cells from undergoing cytokinin-induced cell division.

1.3.6 Conclusions

Both intrinsic and extrinsic signals help to establish polarity in the early plant embryo. The asymmetric zygotic division fixes polarity, which may rely on the asymmetric delivery of cell wall components, possibly AGPs, and which requires *GN* in order to execute it. The fate of the basal cell is now established, and is marked in species as diverse as *Brassica napus* and carrot, by the expression of JIM8-binding AGPs, which may provide cell fate information to the suspensor.

Once the *Arabidopsis* embryo has reached the globular stage, containing roughly 100 cells, the auxin transport mediator *PIN1* becomes polarised in its expression. Again, directional vesicle transport, via *GN*, is required for the correct localisation of the protein within the cell membrane, which is expressed in a polar pattern at the basal end of the cell. The establishment of the auxin transport system is a prerequisite for patterning events in the apical region of the embryo at the beginning of the transition from globular to heart stage embryo. Later in development it is required for hypocotyl and root formation and maintenance, with auxin responsiveness essential in order for the positional information provided by the polar transport of auxin to be interpreted into

pattern elements. Short-range cell-cell communication is required for many of the cell fate decisions, but these clearly depend on the presence of information indicating their position within the apical-basal axis. Regional signalling, involving genes such as *BDL* and other auxin response pathways such as *AXR6* and *MP*, is also crucial to the correct cell division patterns and cell fate decisions which need to occur in the central and basal regions. Analysis of mutants such as *asf1* and *axr6* suggests strongly that auxin signalling is required for the correct cell divisions and cell fate of the suspensor to be established.

Once the meristems in the root and shoot have been established, their self-maintaining ability is determined by the expression of a number of recently discovered genes, although the signalling systems that regulate their expression are far from fully understood. Germination activates the meristems to reiterate the programmes of patterning initiated in the embryo. In the final part of this first chapter we will look at how these signalling mechanisms regulate post-embryonic development through the meristems.

Part Four: Post Embryonic Development

Introduction

Plant embryogenesis is characterised by a balance of oriented cell division and cell expansion. The mature organs are not formed in miniature within the embryo, and so most of what we see is created postembryonically by further cell divisions, differential cell expansion and localised cell differentiation events. Indeed the amount of cell division coupled to the extent of cell expansion creates the size and shape of the organ or tissue, which is in essence how different plants assume their characteristic forms.

Plants develop and maintain groups of stem cells called meristems, which control the development of plant organs through balanced cell proliferation and differentiation. The shoot and root meristems are therefore the major sites of postembryonic cell division. At the shoot apex new organs develop within a specific phyllotaxis, with the aim of increasing the surface area available for photosynthesis and a means for passing on its genes to the next generation. In the root apex the root meristem leads the plant's essential search for water and nutrients within the soil by creating a production line of cells in front of as well as behind itself. In front of the meristem the root cap protects the apex from mechanical damage as it pushes through the soil, whilst behind it the tissues that collect and distribute the water and nutrients to the rest of the plant are formed.

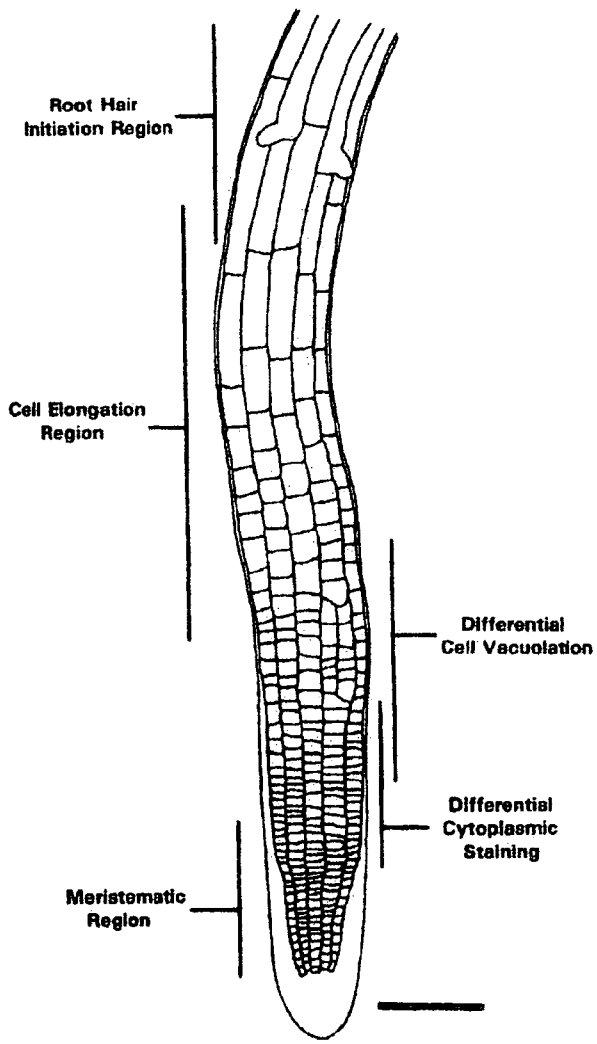
Clearly then it is essential to understand how cell division, cell expansion and cell differentiation events occur, and how they are orchestrated and regulated by different signals in order to understand post embryonic development. In this section we will therefore look at the role of cell division, expansion and differentiation within the postembryonic growth and patterning of the *Arabidopsis* root.

1.4 Growth of the *Arabidopsis* Root

Root growth in *Arabidopsis* is the cumulative result of cell division and cell expansion within the root tip. The growing tip is essentially made up of files of "cellular assembly lines", all involved in the continuous production of the same cell types in approximately the same numbers in the same places (Benfey and Scheifelbein, 1994). Each cell is

therefore developmentally more advanced than the one beneath it. Indeed, the root can be divided into three distinct developmental regions or zones, that can always be seen along the root axis, and that correspond to the processes of cell division, cell expansion and cell differentiation (Esau, 1977). Figure 1.4 below highlights these three regions.

Figure 1.4 The Different Developmental Regions of the Root



Schematic diagram showing longitudinal files of epidermal cells originating at the meristem initials. The developing regions of the root and the location of differences between differentiating root hair and hairless epidermal cells are indicated. The single curved line at the root apex indicates the outline of the root cap.

Bar = 100μm (Masucci and Schiefelbein, 1996).

The three-dimensional architecture of the root is therefore dependent on the interplay of these three key cellular processes. Firstly, the rate and orientation of the plane of cell division within the meristem determines the number of cells added to each file; periclinal divisions create extra radial layers, whilst anticlinal divisions simply add another cell to the file of cells already present in the apical-basal axis. Secondly, the extent and direction of expansion in the elongation zone determines the final shape of each cell, influencing the overall length of and width of the root. Finally, specific cell

differentiation events turn relatively simple cells into units that form the functional tissues of the root. The position of each cell within the radial and apical-basal axis of the three zones determines its developmental state, which is in turn regulated and orchestrated by both local and regional signalling events (van den Berg *et al.*, 1995).

Position is Everything in the *Arabidopsis* Root

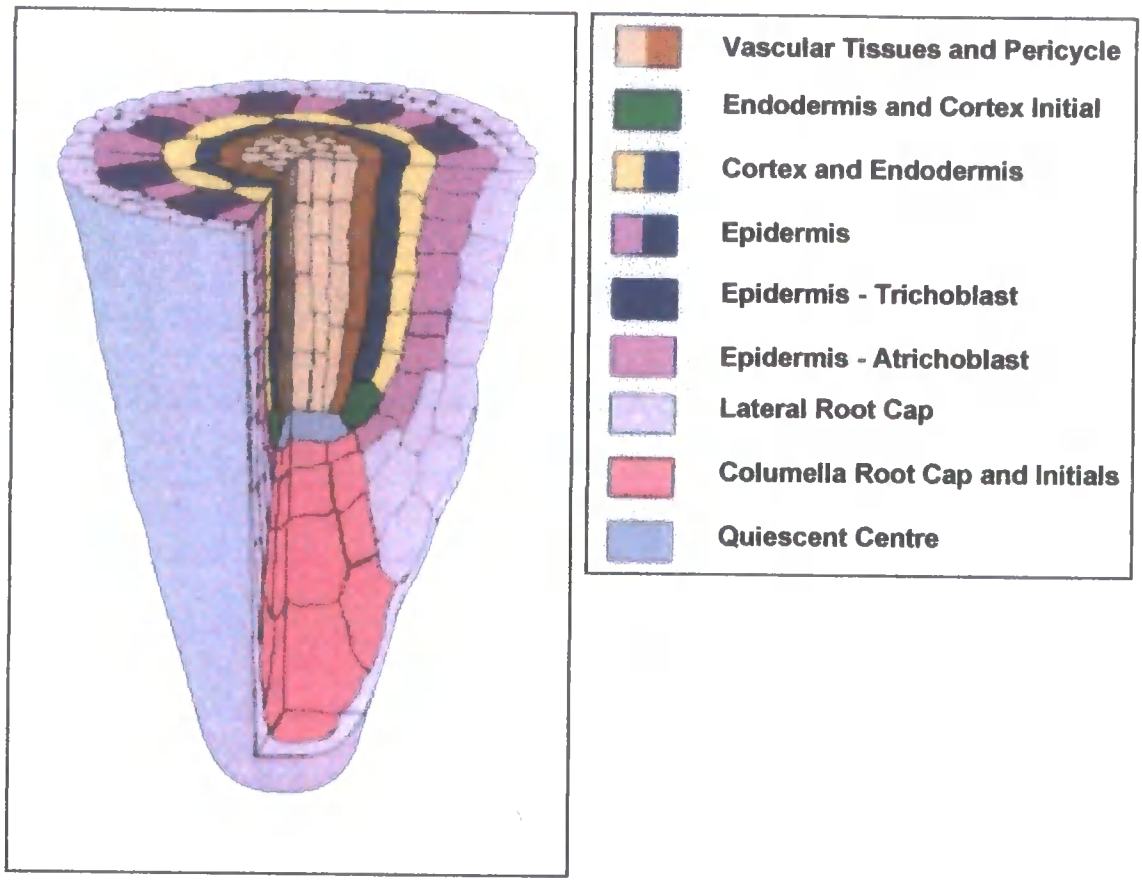
Some elegant work by Claudia van den Berg and colleagues has shown that it is a cell's position in the root that determines its fate, implicating local signalling events in creating the pattern. Using lasers to ablate cells, which are then compressed towards the periphery of the root, neighbouring cells were shown to invade their position and assume their identity (van den Berg *et al.*, 1995). There is a strong correlation between cell type and embryonic lineage, but cell fate requires continuous signalling. When a cortical initial cell was ablated, a pericycle cell invaded its position and divided periclinally to maintain the layers within the root. The pericycle cell lost its suberin staining and then assumed the identity of a cortical initial cell, completing its switch in fate after it had moved across the radial clonal boundary. Similarly, when a epidermal cell was ablated cortical cells invaded and occupied its position. Lineage is therefore not important in the continual patterning of the root, and position is everything. Information guiding cell fate in the radial plane is propagated through an individual cell layer and is directed towards the root tip, with mature cells acting as a patterning template and the initials acting as the source of cells for the continuation of the pattern (van den Berg *et al.*, 1995).

1.4.1 The Meristematic Region

The meristematic region generates the new cells required for the continual growth and patterning of the root. The meristem itself comprises of the quiescent centre (QC) and the dividing cells around it called the initials. The QC is so named because it consists of four non-dividing cells. It is thought that they act as a reservoir of cells so that as the dividing cells around the QC die or lose the potential to divide, a cell within the QC divides to produce two daughter cells, one of which maintains the QC cell fate, whilst the other replaces the old dividing cell (Barlow, 1974). In essence the QC is a set of cells waiting to be activated into division in order to replace the mitotically active cells that surround it. The initials are characteristic of pluripotent animal stem cells, generating a specific tissue(s) by regulated transverse cell divisions.

Together, the entire cluster of initials and the QC make up the "promeristem", which is believed to constitute the minimal group of root meristem cells (Clowes, 1961).

Figure 1.4.1 The Root Meristem



(Figure taken from http://www.bio.uu.nl/~mcbroots/anatmap.htm)

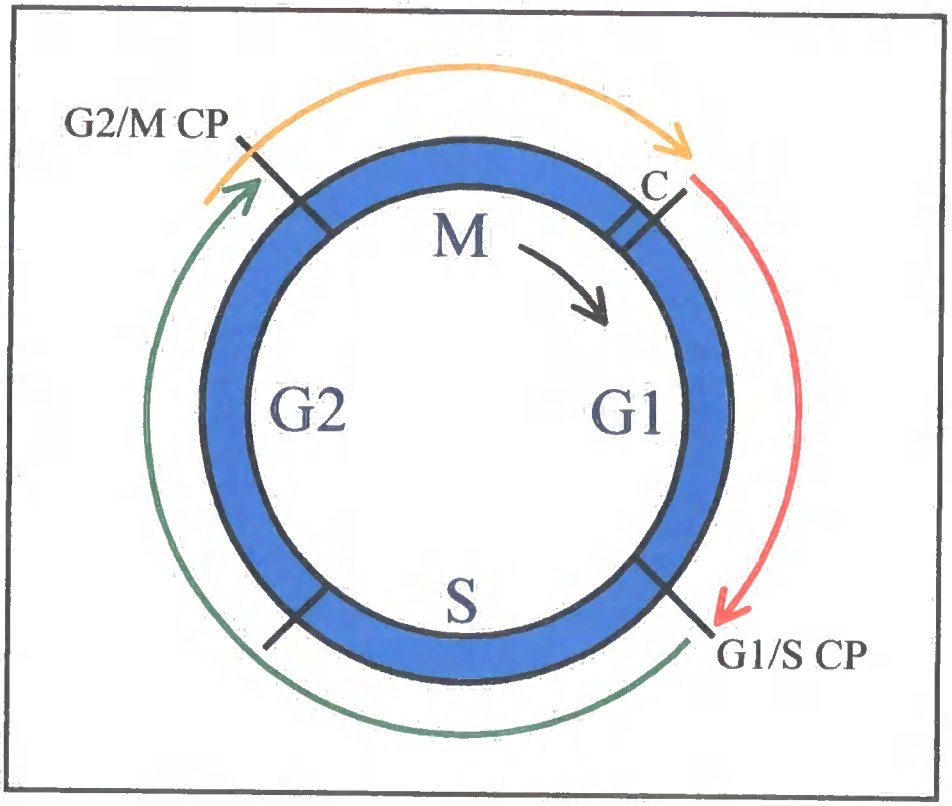
Recent evidence has led to a greater understanding of the role of the QC in regulating the pattern within the meristem around it. Laser ablation of QC cells leads to the differentiation of the initial cells that abut the dead cell (van den Berg *et al.*, 1997). The initials are mitotically active, producing daughter cells that then start to undergo fate –specific divisions and differentiation events. However ablation of the underlying QC cell results in cessation of cell division, and the cell then assumes the identity of its daughter cell, an event shown previously to be directed by information from the mature cells within that cell file (van den Berg *et al.*, 1995). In the *hobbit* (*hbt*) mutant all of the cortical cells divide into cortex and endodermis during embryogenesis, indicating that a functional QC is required for the initials to maintain their status (Willemsen *et al.*, 1998). Together these results show that the QC inhibits the differentiation of the surrounding initial cells within a single cell range. The pattern generated by the

meristem is therefore maintained by a combination of short range inhibition and reinforcement of cell fate decisions by the mature cells within the file (van den Berg *et al.*, 1997; van den Berg *et al.*, 1995).

The Cell Cycle

Dividing cells move through the cell cycle at a faster rate when compared with non-dividing cells, and this is achieved by regulation at the G1/S and G2/M transition checkpoints. The progression of the cell cycle machinery past these two checkpoints is regulated by the combined interaction of specific cyclins with specific cyclin-dependent kinases (CDKs) (Evans *et al.*, 1983). Passage through the checkpoints requires the activation of CDKs, and this is achieved by their association with cyclins and by altering the phosphorylation state of the associated CDK (Pines, 1993). However, whilst CDKs are expressed throughout the cell cycle in dividing cells, the expression of cyclins fluctuates depending on its position in the cell cycle (Doerner *et al.*, 1996; Fobert *et al.*, 1994; Hemerly *et al.*, 1992, 1993; Martinez *et al.*, 1992).

Figure 1.4.1.2 The Cell Cycle and Cyclin Expression



The cell cycle is divided into four stages, Mitosis (M) which ends with cytokinesis (C), post mitosis Interphase (G1), DNA synthesis (S), and post-synthetic Interphase (G2). The two major checkpoints are labelled (G1/S CP and G2/M CP). Cyclins are expressed

throughout specific stages, A type cyclins (green arrow), B type cyclins (orange arrow), and D type cyclins (red arrow).

Interestingly the different classes of cyclins appear to regulate the same cell cycle controls and checkpoints in both animals and plants (The *Arabidopsis* Genome Initiative, 2000). They are divided into mitotic cyclins (A and B types) and G1 cyclins (D type). Specifically, A type cyclins are expressed from S phase and are destroyed by the end of M phase, whilst B type cyclins accumulate only during late G2 phase but are also destroyed at the end of mitosis. Both the A and B type cyclins contain a conserved nine amino acids 'destruction box' motif at their N-terminus that is homologous to those of mitotic cyclins from animals and fungi (den Boer and Murray, 2000; Renaudin *et al.*, 1996). The destruction box targets the cycle for degradation by ubiquitin-mediated proteolysis via the anaphase-promoting complex (APC) (den Boer and Murray, 2000).

Manipulation of the Cell Cycle and Plant Growth

With the identification of cell cycle genes in *Arabidopsis*, molecular genetic techniques have been used to alter cell cycle progression in transgenic plants. Increasing the amount of cell division in the root by up-regulation of *cyc1At* in transgenic *Arabidopsis* caused a marked acceleration in the growth rate without altering the patterning of the root itself (Doerner *et al.*,1996). Alternatively, Hemerly and colleagues (2000) have used a dominant negative version of *CDC2a* that stops cells progressing through the cycle. Expression of this recombinant protein under the control of *At252* albumin gene promoter was used to investigate the influence of reduced cell division upon embryogenesis. The promoter is active from the late globular stage onwards, and is ubiquitously expressed. By the late heart stage most of the pattern elements are present and so the main effects were restricted to the size of the organs and the amount of cells within the future meristems. Reduced cell divisions in the apical region of the embryo caused a reduction in the size of the cotyledons, which were also fused and exhibited phyllotactic problems, whilst in the root the root meristem and embryonic root were simply reduced in size (Hemerly *et al.*, 2000).

These observations show that the cell cycle must be regulated in order for the plant to attain the correct size and shape of the organs, and it is this link between overall growth rate and cell cycle progression that is crucial if we are to see how hormones influence root growth and development.

Hormonal Control of the Cell Cycle

A number of major plant hormones have been implicated in influencing cell cycle progression and the activity of the root meristem, however we will focus here on the influences of auxin, cytokinin, BRs and ethylene.

The promotion of cell division activity by plant hormones is reflected in the hormonally induced activities of CDKs, in the level of their mRNAs, and also in the level of cyclin transcripts. For example, John *et al.* (1993) showed that auxin alone increased the level of a $p34^{cdc2}$-like protein kinase in cultured tobacco cells, but addition of a cytokinin was required for activation of this kinase. Indeed, it is well known that cytokinin is required in concert with auxin for cell division in cells cultures, and there is now considerable evidence that cytokinin plays an active role in stimulating cell division *in vivo*. Immunocytochemistry and direct measurements of cytokinin levels reveal high amounts in meristematically active cells and reduced amounts in mitotically inactive cells (Mok and Mok, 1994 and 2001; Dewitte *et al.*, 1999).

Transgenic tobacco plants expressing four homologues of the *Arabidopsis* cytokinin oxidase genes, enzymes that degrade cytokinin, under the CaMV 35S promoter showed considerable reduction in growth. This was shown to be due to reduced cell division within the shoot meristem and was linked to reduced levels of endogenous cytokinins (Werner *et al.*, 2001). Interestingly though, this reduction in cell division was only seen in the shoot, where as there was an increase in overall root growth and cell division in the root meristem, suggesting that cytokinin may have opposing effects in shoot and root development. Also, the knock-on effect of the altered ratio of cytokinin to other hormones, such as auxin, must be taken in to account here when interpreting this phenotype. However these observations are consistent with cytokinins having a role in regulating cell division *in vivo*.

Recent evidence shows that cytokinin regulates the G1/S transition through induction of one of the three D-type cyclins present in *Arabidopsis*. *CycD3* was induced in cell cultures within one hour of reintroduction of cytokinin to cells starved of cytokinin for twenty-four hours. Also, cell lines over-expressing the *cycD3* gene were found to callus without the need for cytokinin in the medium. (Riou-Khamlichi *et al.*, 1999; Soni *et al.*, 1995).

The involvement of cytokinins at the G2/M phase transition has been shown in cultured *Nicotiana plumbaginofolia* cells (Zhang *et al.*, 1996). Cells that arrested in G2 because of a lack of cytokinin accumulated high levels of phosphorylated *Cdc2*, which is the inactive state of the major CDK at this checkpoint (Zhang *et al.*, 1996). The immediate consequence of cytokinin reintroduction (0.23μM kinetin) was entry into M phase and dephosphorylation of *Cdc2*. Subsequently, the same workers observed that this dephosphorylation could also be mediated by fission yeast *Cdc25 phosphatase*.

A picture therefore emerges of cytokinin encouraging the progression of cells through the cell cycle through the up-regulation of *cycD3* at the G1 checkpoint and the phosphoregulation of the CDK *Cdc2* at the G2/M checkpoint.

Experiments involving the expression of *ABP1* have yielded indications of auxin's role in regulating the cell cycle. *ABP1* over expressing tobacco leaves showed double the amount of cells at the G2 stage when compared with wild type, however auxin induced cell expansion occurred before this advance in the cell cycle, and so this may have been an indirect effect of a change in cell volume (Chen *et al.*, 2001). However, a transgenic BY2 cell line carrying a tetracyclin inducible *ABP1* specific single short chain antibody fragment, ScFV12, which binds *ABP1 in planta* and alters its ability to function, caused no change in cell volume but arrested the cells in G1, an effect that was reversed once tetracyclin was washed off (C. Perrot-Rechenmann, personal communication).

Therefore auxin and *ABP1* may have a role in regulating the cell cycle at the G1 and G2/M check points, although this still needs some clarification. One possible link between *ABP1* and the cell cycle is through the membrane associated heterotrimeric G proteins. *Arabidopsis* cells overproducing a heterotrimeric Gα protein mimicked an auxin induced increase in cell division (Ullah *et al.*, 2001). Also, the expression patterns of the Gα gene localise to the meristem regions, consistent with a role in cell division regulation (Ma *et al.*, 1990). It has recently been proposed that auxin activates cell division not via *ABP1* by through another as yet unidentified lower-affinity binding protein coupled to heterotrimeric G protein signalling (Chen, 2001). BY2 cells undergo maximal auxin-induced cell expansion at 0.3μM IAA, and maximal cell division at 30μM IAA, implementing a high affinity binding protein (*ABP1*) in cell expansion, and a low-affinity binding protein (?) in auxin induced cell division. The *altered lateral root*

formation4 (*alf4*) mutant shows an uncoupling of auxin induced cell division and expansion, lateral root induction by auxin stimulated cell divisions in the pericycle is lost, however inhibition of root elongation by high auxin concentrations is still seen (Celenza *et al.*, 1995).

Presently, the evidence for *ABP1*'s role in cell expansion is more abundant than it is for regulating cell division, and since the major evidence for its role in cell division has not been subjected to peer review, it must be treated with caution. The possibility of a second auxin signalling pathway that cross-talks with *ABP1* mediated cell expansion is a new concept, and one that does not have much direct evidence, and so at this stage can only be considered an interesting hypothesis for future investigation (Chen, 2001).

Kerk and Feldman (1995) have proposed a indirect biochemical model for auxin's role in initiating and maintaining the quiescent centre of the maize root meristem. The QC is located at the distal part of the root, and is also the most distant tissue from the path of polar auxin transport. Ascorbic acid (AA) is a compound which is necessary for the transition from the G1 to the S phase in the cell cycle, and which is broken down by ascorbic acid oxidase (AAO). AAO mRNA is increased in response to auxin, which was shown to have higher levels in the quiescent centre than surrounding cells, determined by immunolocalisation of auxin in the root tip. The authors propose that auxin is influencing AAO levels within the root meristem, and that this ensures the continued stem cell ability of the quiescent centre. Recently they have also shown that AAO can oxidatively carboxylate IAA, which is a recognised pathway for degradation of auxin, but this only occurs in the presence of the root cap (Kerk *et al.*, 2000). Auxin therefore increases AAO levels, which reduces AA levels and maintains the quiescent nature of the cells, and then it feeds back upon itself by decreasing auxin levels through oxidative decarboxylation.

The influence of auxin on the activity of the root meristem is also elegantly demonstrated through studies by Sabatini and co-workers (1999). The authors utilised a synthetic auxin-responsive promoter construct, termed *DR5*, which consists of 7 tandem repeats of an auxin-responsive element fused to the β-glucuronidase (GUS) reporter gene (Ulmasov *et al.*, 1997a). The *DR5* reporter is activated rapidly by auxins within the 10^{-8} - 10^{-4} M range. Expression of this gene fusion shows a 'maximum' in the root meristem region, in the columella initials of wild-type seedlings. By studying the effect

of known mutations on the position of the auxin 'maximum', they suggest that pattern and polarity in the *Arabidopsis* root is mediated by the auxin maximum, located distal to the vascular tissue boundary.

Therefore, auxin induced AAO may play a role in maintaining the identity of the QC, and the auxin 'maximum' may allow the maintenance of meristem activity, which sustains the pattern of the root.

BRs have previously only been implicated in regulating cell expansion, indeed, both BR deficient and insensitive mutants indicated that the dwarf phenotype resulted from a reduction in cell size rather than in cell number (Kauschmann *et al.*, 1996). However recent evidence has shown that brassinolide up-regulates *cycD3* levels through *de novo* protein synthesis, but does not alter protein phosphorylation or dephosphorylation (Hu *et al.*, 2000). Induction was also found to occur in *bril* mutants, implying that the induction of *cycD3* occurs not via the membrane-bound receptor but by another mechanism, possibly an intracellular signal pathway in a similar fashion to mammalian steroid hormones.

1.4.2 The Cell Expansion Region

Once the cell has exited the cell cycle, possibly due to its new position within the peripheral regions of the developing meristem and outside the influence of the QC, the cell starts to undergo rapid expansion; before they mature plant cells enlarge between 10- to 1000-times in volume. They accomplish this through water uptake and irreversible cell wall expansion, which is regulated in a highly cell-specific manner (Cosgrove, 1997). Plant cell expansion is also regulated co-ordinately at the whole-organ level by environmental stimuli such as light, temperature, gravity, and water availability. Such environmental triggers influence the levels of hormones so that the plant can respond to changes in its environment.

Cell size and shape is ultimately governed by the mechanics of the cell wall, which can be visualised as a thin, fibrous layer strong enough to withstand the high physical stresses generated by cell turgor pressure (Cosgrove, 1993). Therefore, the critical event required for cell enlargement is the loosening or stress relaxation of the wall. This is the

process by which growing cells simultaneously loosen their walls and reduce turgor and water potential, so as to allow water uptake and physical expansion (Cosgrove, 1993).

Once wall loosening has occurred, the elongation process begins and is driven by the uptake of water into the cell, that results from increased water potential as brought about by wall relaxation. The water in the cell pushes against the newly loosened walls and causes expansion in a certain direction, determined by the orientation of the cellulose microfibrils that make up the wall, which in turn are influenced by the orientation of the cortical microtubules (Giddings and Staehelin, 1988). The microtubule network connects to the cellulose synthase complex, forming a hexameric rosette structure that is present in the plasma membrane. Inhibition of either microtubule polymerisation or cellulose synthase activity leads to a cessation of cell expansion (Nicol and Hofte, 1998).

As walls expand they do not appear to become thinner, but instead manage to maintain the same thickness. Wall synthesis is therefore thought to be a well co-ordinated and integral part of the expansion process. Hemicellulose and pectin are delivered to the plasma membrane from the Golgi body by directed vesicle transport, where it is thought that the cellulose microfibrils are made and deposited into the extracellular space, where further enzymes help to integrate the new fibril into the wall. Whether the formation of the new fibril by the cellulose synthase drives the movement around the cell, or the cortical microtubules guides it is still a matter of debate.

Elongation can only occur in the plane that is at a right angle to the length of the microfibril, so the orientation of the microfibrils in the wall offers the cell a point at which to exert control over the direction of expansion. It is thought that fully differentiated cells that have reached their full size and shape restrict any further expansion by orientating their cellulose microfibrils accordingly (Martin *et al.*, 2001). Also, enzymes such as pectin methylesterases, peroxidases and other cross-linking enzymes lock up the wall during its maturation, so preventing further wall creep.

Recently *Arabidopsis* mutants have been isolated that are defective in cell expansion, such as the conditional root expansion mutants (CORE), *pompom1*, *pompom2*, *lion's tail*, *cobra*, *cudge* and *quill* (Hauser et al., 1995). It is envisaged that these mutants will be defective in genes encoding key regulatory elements of the cell expansion

machinery. One mutant gene that has been cloned is *root swelling1* (*rsw1*). *RSW1* encodes a122 kD protein that has homology to bacterial cellulose synthases, and is thought to be one of the catalytic subunits of the cellulose synthase complex (Williamson *et al.*, 2001). It contains 6 trans-membrane domains and a conserved UDP-glucose binding motif, UDP-glucose is the precursor for cellulose, as well as a protein-protein interaction motif. There are presently 17 identified *RSW1* homologues within the *Arabidopsis* genome.

Hormones and Cell Expansion

Hormones have been implicated in influencing the direction of cell expansion through the orientation of the microtubules and therefore the cellulose microfibrils within the wall. Induction of cell elongation by gibberellins is confined to meristematic and young cells because their cellulose microfibrils are orientated transversely, whilst auxin, which is known to cause a reorientation of cellulose microfibril deposition from the oblique/longitudinal to the transverse, is able to promote the elongation of cells that have stopped growing (Bergfeld *et al.*, 1988). Ethylene is known to inhibit the elongation of terrestrial plants and cause the thickening of their stems. This effect has been ascribed to a reorientation of the cortical microtubules from mostly transverse to mostly oblique/longitudinal (Lang *et al.*, 1982; Roberts *et al.*, 1985).

Hormones that modulate the rate of cell expansion usually do so by influencing cell wall properties, such as auxin causing acidification of the cell wall. The acid-growth theory postulates that secretion of hydrogen ions into the cell wall is stimulated by auxin and that the lowered pH in the apoplast activates wall-loosening processes (Rayle and Cleland, 1970). There are several lines of evidence that support this hypothesis, in particular, the fact that auxin causes extrusion of H^+ ions by H^+ATPases, which acidifies the cell wall space. Indeed, antibodies designed to stimulate the putative auxin receptor *ABP1* showed that membrane hyperpolarization is the earliest event in auxin-induced cell wall loosening (Barbier-Brygoo *et al.*, 1989 and 1991; Ruck *et al.*, 1993; Thiel *et al.*, 1993; LeBlanc *et al.*, 1999). Furthermore, ectopic and inducible expression of *ABP1* confers auxin dependent cell expansion in cells normally lacking auxin responsiveness (Jones *et al.*, 1998), and the *abp1* mutant phenotype confirms a role in mediating cell expansion *in vivo* (Chen *et al.*, 2001). Acidic buffers can also induce growth in auxin-insensitive tissues (Rayle and Cleland, 1992).

It is thought that changes in the pH of the cell wall alter the activity of the enzymes involved in loosening the cell wall, such as expansins, xyloglucan endo-transglycosylases (XETs) and endo-1,4-β-D-glucanases, as proteases and protein-denaturing treatments eliminate the acid-extension response (Cosgrove, 1989). Expansins are a family of proteins that exhibit wall-loosening activity at around pH 4.5 (Cosgrove, 1996 and 1997), they are 25-27 KD in size and have homology to a family of fungal glycosyl hydrolases (family-45 glucanases). Non-growing cells do not respond to expansins, suggesting that as walls mature they lose their ability to respond to expansin action. Indeed, it is thought that the primary wall is initially secreted and assembled in a form that is mechanically tough yet has "hot spots" where expansin can weaken microfibril-matrix adhesion (Cosgrove, 1997). It has been proposed that expansins cause wall creep by loosening non-covalent associations between wall polysaccharides, and that this is modulated by both the secretion of the protein to the wall and through changes in the pH and redox potential of the wall itself. However, not only is the exact action of expansin on the wall polymers not known, it is made even more confusing by the fact that the family-45 glucanases do not cause wall creep (Cosgrove, 1993).

Recent evidence has shown a way in which the acid growth hypothesis and expansin driven wall loosening can be integrated. Localised addition of auxin and expansins to shoot apical meristems stimulates leaf outgrowth (Fleming *et al.*, 1997; Reinhardt *et al.*, 2000), and *in situ* hybridisation shows that auxin-inducible expansin transcripts accumulate at the presumptive sites of leaf initiation (Reinhardt *et al.*, 2000; Caderas *et al.*, 2000). It is also possible that auxin could regulate expansin activity at the post-translational level through its effects on cell wall pH (Cosgrove, 1996).

A comparison of BR and auxin-promoted elongation shows that the kinetics of BR-promoted elongation are slower than those of auxin, but that BR is more effective than auxin in promoting elongation after long incubations (Clouse *et al.*, 1992). Also, in contrast to auxin, BR does not rapidly induce members of the auxin-inducible gene families prior to the onset of elongation. This apparent independence of auxin and BR-promoted elongation is further supported by the findings that BR exerts the same physiological effects on auxin-insensitive mutants of tomato and *Arabidopsis* as it does the corresponding wild-type plants (Clouse *et al.*, 1993; Zurek *et al.*, 1994).

It appears that, like auxin-induced cell elongation, BR induced cell elongation is ultimately mediated by cell wall loosening. One BR-stimulated gene that has been cloned, *BRASSINOSTEROID UP-REGULATED 1* (*BRU1*) shows extensive homology to numerous XET encoding genes from mung bean, soybean and tomato (Arrowsmith and de Silva, 1995; Okazawa *et al.*, 1993; de Silva *et al.*, 1993), and recombinant *BRU1* protein has been shown to have XET activity (Clouse *et al.*, 1996). Also, in *Arabidopsis* there are at least seven other XET or XET-related sequences, of which, *TCH4* has been shown to encode an active XET that is regulated by BRs (Xu *et al.*, 1995). Applications of 1.0μM BR causes an increase in *TCH4* transcripts within 30 minutes with a maximum at 2 hours (Xu *et al.*, 1995).

XETs are enzymes that have a proposed wall-loosening role; one of the major wall polymers is xyloglucan and XETs cut the backbone of xyloglucans, joining the newly formed reducing end to the non-reducing end of an acceptor xyloglucan, thus lengthening the xyloglucan polymers and therefore the wall. XETs are thought to affect the amount of wall enlargement that results from expansin activity (Cosgrove, 1997). XET also has other important roles in elongation such as xyloglucan biosynthesis or integration of new xyloglucan polymers into the wall (Fanutti *et al.*, 1993; Fry *et al.*, 1992; Nishitani and Tominaga, 1992; McQueen-Mason *et al.*, 1993).

The recently cloned *KORRIGAN* (*KOR*) gene encodes one member of the *Arabidopsis* endo-1,4-β-D-glucanase family, of which there are at least 10 members (Nicol *et al.*, 1998). The *KOR* protein is membrane bound and localised predominantly at the plasma membrane as well as some being present in the Golgi. The *kor* mutation affects all cell types except those undergoing tip growth, with cells showing reduced elongation and increased radial expansion. The family exhibits tight temporal, spatial and hormonal regulation, indicating an important role for these genes in development. One group is ethylene-inducible and correlated with massive wall degradation during fruit ripening and leaf abscission (Brummel *et al.*, 1997). A second class of these enzymes appears to be correlated with more subtle processes involved in the rearrangement of polysaccharides in growing cells, and so implicates a role in contributing to the loosening of the wall and the incorporation of new microfibrils into the growing wall (Nicol and Hofte, 1998). The *kor* mutants have an unordered accumulation of cellulosic material at the cell wall surface, and this observation supports such a role in incorporation as well as modification of microfibrils. However the levels of the mRNA

of the tomato *KOR* homologue, *CEL3*, have been found not to be influenced by either BRs, auxin or ethylene (Brummel *et al.*, 1997). Although there does not appear to be a role for this particular gene in hormonally induced cell expansion, other members of the family are possibly involved, but more work needs to be done in this area.

Auxin and Differential Cell Expansion: The Gravity Response

Roots are positively gravitropic whilst shoots are negatively gravitropic but positively phototropic. This ensures that the plant finds itself with its roots in the ground and its leaves above the ground and facing the sun so as to be able to maximise its photosynthetic potential. Clearly plants cannot move like animals as they do not possess muscles, so they have to achieve movement through a process of differential growth, which must obviously directed by the external signal. Charles Darwin was the first to discuss the tropic growth of plants, and he implicated the differential distribution of a signal which induces the expansion of cells on one side of the stem or root, such that it bends towards the stimulus, although he did not know what this signal was (Darwin, 1881). We now know that this signal is auxin, and with our increased understanding of auxin signalling and the possibility of growing plants in space (zero-gravity), the gravity response in particular has come under renewed investigation.

The primary site for gravity sensing in the root is the root cap columella, which is formed of three tiers of starch-containing cells. The first row of columella cells distal to the columella initials is referred to as the S1 layer, and the subsequent rows as S2 and S3. Laser ablation experiments have shown that the both the S1 and S2 layers are important in the gravity response (Blancflor *et al.*, 1998). The columella cells, or statocytes, are highly specialised. The nucleus is located at the top of the cell, the endoplasmic reticulum at the periphery, and the starch-filled amyloplasts, or statoliths, sediment to the physical bottom of the cell (Rosen *et al.*, 1999). The statoliths have recently been shown to be enmeshed within the actin cytoskeleton (Collings *et al.*, 2001). Upon gravistimulation, actin-enmeshed statoliths sediment from their position prior to the stimulus to the new physical bottom of the cell. The starch-statolith hypothesis proposes that amyloplast sedimentation is the primary gravity-sensing mechanism (Haberlandt, 1900; Nemec, 1900), whereas the gravitational pressure model proposes that statocytes perceive gravity by sensing the tension and pressure that exists between the plasma membrane and its extracellular matrix on opposing flanks of the

cell (Staves, 1997). Whatever the mechanism, it appears that starch granules are essential, as starch mutants have poor gravity responses (Kiss *et al.*, 1996).

There is currently little knowledge of how the transduction of the signal indicating that the gravity vector has changed, is achieved. What is clear though, is that it brings about a change in auxin distribution across the root tip. This is achieved through the relocation of specific transport proteins, both influx and efflux carriers. As early as 1926 the Cholodny-Went theory proposed that gravistimulation promotes the development of an auxin gradient across the root tip, and that this is responsible for the differential rate of cellular elongation on opposite flanks of the root (Went, 1974). With the cloning of the genes encoding the influx and efflux carriers of auxin, the proteins that determine the direction of auxin through a tissue, we are now able to put a molecular model onto this theoretical framework.

The influx carrier *AUX1* is specifically localised within the S2 layer of the root cap columella, which has been shown to be crucial in the perception of the gravity response (Blancflor *et al.*, 1998). *aux1* mutants are agravitropic and also show a reduced amount of auxin in the root tip, which establishes a sharp gradient within wild type seedlings, with the highest amount seen within the most apical 1mm of the tip (). Mutants also showed reduced expression of the *IAA2::GUS* reporter gene within the proximal and central elongation zones of the root, indicating that *AUX1* is required for the basipetal transport of auxin and is part of the gravity response machinery (Swarup *et al.*, 2001).

The PIN proteins exhibit specific localisation within each cell types in which they are found. They cycle rapidly between the plasma membrane and an undefined endosomal compartment, a process that requires functional *GNOM* protein and is actin-dependent (Geldner *et al.*, 2000). PIN3 protein is localised uniformly around the columella root cap cells, specifically the S1 and S2 layers, and *pin3* mutants were found to exhibit an agravitropic phenotype (Friml *et al.*, 2002b). Upon gravistimulation PIN3 becomes rapidly re-localised, which is able to occur due to the rapid cycling of the proteins. Within 2 minutes of altering the gravity vector PIN3 protein was detected in an asymmetric distribution at the lateral side of the cell, which was completed within 5 minutes and maintained up to 20 minutes after stimulation. 1 hour after gravistimulation the asymmetry of PIN3 localisation was seen at the tissue level, with protein detected asymmetrically across the root tip in the columella initials and the lateral root cap

(Friml *et al.*, 2002b). The relocation of PIN3 within the S1 and S2 layers could be brought about by the actin-enmeshed statoliths sedimenting to the new physical bottom of the cell, causing a knock-on effect upon the actin cytoskeleton. The actin-dependent delivery of the PIN3 protein to the cell membrane would therefore be altered.

A second *pin* mutant, *pin2/eir1/agr1*, has been isolated that shows an agravitropic phenotype (Muller *et al.*, 1998; Luschnig *et al.*, 1998; Chen *et al.*, 1998). The *PIN2* protein is localised within the lateral root cap and the cortex and epidermis within the proximal and central elongation zones (Muller *et al.*, 1998; J. Friml, unpublished data, personal communication). Similar to *aux1* mutants, *eir1* mutants also show reduced expression of *IAA2::GUS* within this region. Within wild type seedlings the *IAA2::GUS* reporter shows an asymmetric staining pattern after gravistimulation, which is not seen within *eir1* mutants, indicating that the asymmetric relocation of auxin into the proximal and central elongation zones is essential for the differential growth response (Luschnig *et al.*, 1998).

Mutations in *AXR3*, the *AUX/IAA17* gene, also cause an agravitropic root phenotype, indicating that the differentially distributed auxin requires perception and signal transduction pathways leading to the degradation of the AUX/IAA transcriptional repressors, and the subsequent activation of gene expression (Leyser *et al.*, 1996). The *axr3-1* mutation represents a domain II mutation, causing a stability of the protein that is expressed in the root elongation zone (K. Knox and O. Leyser, York University, personal communication). The wild type degradation of the *AXR3* protein allows transcription of downstream auxin genes, however in the *axr3-1* mutants the protein is seven times more stable and so it is unable to allow the activation of downstream gene targets within this zone (Ouellet *et al.*, 2001).

Together these results support the starch-statolith model of graviperception and the Cholodny-Went hypothesis of auxin-mediated tropic growth.

1.4.3 The Region of Cell Differentiation

As cells are continually produced by the meristem there is a constant supply of cells entering the zone of elongation, and consequently reaching the zone of differentiation. The establishment of this 'production line' of cells is important for the correct

patterning of the root, as directional signalling from the more differentiated cells within a cell file initiate cell fate processes in the less differentiated cells behind them (van den Berg *et al.*, 1995). These signals may be restricted to a specific cell layer through a plasmodesmal network, which has the greatest connectivity between cells within the same layer (Scheres, 1997), and in the epidermis it has been shown to persist until the cells are fully differentiated (Duckett *et al.*, 1994).

1.4.3.1. Radial Patterning: Specification of the Cortex and Endodermis

The two cell layers that make up the ground tissue, the cortex and endodermis, originate from one initial cell, that divides anticlinally to produce a daughter cell that then undergoes an asymmetric periclinal division. The periclinal division creates an thinner inner cell layer, the future endodermis, and a wider outer cell layer, the future cortex. Two genes have been identified that control these events and the subsequent differentiation events that the two layers undergo, and they were isolated through a conventional mutant screen, for radial patterning mutants (Benfey *et al.*, 1993; Scheres *et al.*, 1995). Both the *shortroot* (*shr*) and *scarecrow* (*scr*) mutations cause a only one layer to be present rather than both cortex and endodermis. *shr* mutants contain one ground tissue layer that lacks endodermal markers, suggesting that it is required for the periclinal division that generates these two layers, but also that it is required for the specification of the endodermal cell fate (Helariutta *et al.*, 2000). In support of this, *SHR* has been shown to encode a transcription factor that is similar to the GRAS family, and ectopic expression causes supernumerary cell divisions and altered cell specification (Helariutta *et al.*, 2000). *SCR* also encodes a transcription factor that is also a member of the GRAS family, however *scr* mutations lead to one ground tissue layer that expresses markers of both the endodermis and cortex, indicating that it is also required for the asymmetric division that generates the two ground layers, but unlike *SHR* that it is not involved in the specification of cell fate (Di Laurenzo *et al.*, 1996; Wysocka-Diller *et al.*, 2000).

Both mutations are evident in the embryonic axis (Scheres *et al.*, 1995), and expression of *SHR* is seen from the globular stage onwards, with expression in the procambium (the vascular precursors) and the lower tier. *SCR*, however is localised in the ground tissue and the hypophysis at this stage. By the triangular stage *SHR* expression is restricted to the procambium, where is seen for the rest of development, whilst *SCR*

continues to be expressed in the adjacent layers (ground tissue) as well as the QC (Helariutta *et al.*, 2000; Wysocka-Diller *et al.*, 2000). In the seedling root *SCR* is expressed in the initial daughter cell before the asymmetric division and then remains expressed in the endodermal call layer after this division (Di Laurenzo *et al.*, 1996; Wysocka-Diller *et al.*, 2000), whilst *SHR* is restricted to the pericycle and the vascular cylinder adjacent to the ground tissue indicating that it acts in a non-cell-autonomous manner (Helariutta *et al.*, 2000).

The finding that *SHR* mRNA was localised in a different cell layer to which it was believed to work, the ground tissue layers, implicated lateral movement of the *SHR* transcription factor. The *KNOTTED1* maize transcription factor has been shown to move from one cell lineage to another (Lucas *et al.*, 1995), and so it was a testable hypothesis that *SHR* may also move. An inframe *SHR-GFP* fusion protein was made and *Arabidopsis* seedlings transformed with the construct. The localisation of the *GFP* signal was confirmed with *GFP*-specific antibodies. Expression was seen in the nucleus of the cells adjacent to those in which *SHR* mRNA was localised, indicating that it had moved, possibly through a plasmodesmal connection (Nakajima *et al.*, 2001). *SHR* is required for *SCR* expression (Helariutta *et al.*, 2000), and so it is thought that *SHR* moves the nucleus of the adjacent cell to activate *SCR* expression, as well as other genes required for the specification of the endodermis. To achieve this the protein may have a short half-life and so only be able to influence the adjacent cell layer and not act further than this. This work highlights the importance of local signalling events within the radial plane and implicates a role for plasmodesmal cell-cell connections in patterning of the root.

Two other interesting genes have been identified through a mutant screen, however this was designed to isolate genes involved in the patterning of the epidermal layer (Cnops *et al.*, 1996). The lateral root cap and epidermis are derived from the outermost tissue layer of the embryo, the protoderm (Dolan *et al.*, 1993; Scheres *et al.*, 1995). Postembryonically these tissues derive from a ring of sixteen root cap and epidermis initials at the root tip, that divide periclinally to form a pair of daughter cells. The outer cell follows the lateral root cap fate, whilst the inner cell undergoes anticlinal divisions and follow the epidermal cell fate. Both sets of cells divide within the meristematic zone, however by the time they reach the elongation zone the root cap cells die (Cnops *et al.*, 2000).

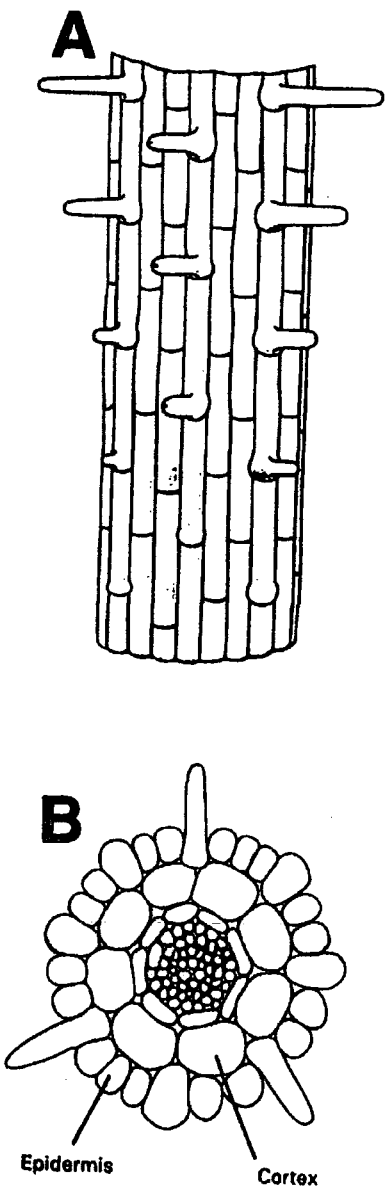
The *tornado* (*tor*) mutants fall into two complementation groups, designated *tor1* and *tor2*, double mutants of which show a single phenotype and so indicate that the genes act in the same pathway (Cnops *et al.*, 2000). The mutant phenotypes implicate both *TOR* genes in specifying the fate of the cells derived from the protoderm, although they are not required embryonically. Mutant roots are shorter and twisted with ectopic specification of both root hair and non-hair cells. They therefore appear to be negative regulators of root cap cell fate among cells in the epidermal cell position, and are required for the correct patterning of root hairs. However neither gene has so far been cloned, and so a more precise understanding of how these genes work awaits further research. It is interesting to speculate though, as *TOR1* has been shown to be allelic to *LOP1*, which is also uncloned but which has been shown to have reduced auxin transport in stem sections (Carland and McHale, 1996; Cnops *et al.*, 2000). Therefore auxin transport may have a role in regulating patterning information within the epidermis, and the localisation of *PIN2* within the lateral root cap and the epidermis supports this hypothesis (J.Friml, unpublished data, personal communication).

1.4.3.2 Circumferential Patterning: Root Hair Development

Arabidopsis has only two cell types in the root epidermis, root hair cells, which are derived from trichoblasts, and hairless cells, which are derived from atrichoblasts. Figure 1.4.3.2 below shows the organised and regular pattern of root hair development in *Arabidopsis*. One of the first steps therefore in epidermal cell differentiation is fate specification; whether the cell adopts the root hair or the hairless cell fate. In *Arabidopsis*, differential cell identity is established by the late torpedo stage of embryogenesis, and is maintained in the seedling by positional information (Berger *et al.*, 1998; van den Berg *et al.*, 1995); trichoblasts form in the crevice between underlying cortical cells (outside an anticlinal cortical cell wall), and atrichoblasts develop over the cortical cell (outside a periclinal cortical cell wall). The precursors of the two mature cell types can be distinguished from one another throughout their differentiation (Dolan *et al.*, 1994; Galway *et al.*, 1994). The earliest distinctions are apparent from when the cells are in the meristematic region, where they show differences in cytoplasmic density, with root hairs having denser cytoplasm. During later stages the two cell types also differ in their rate of vacuolation, their extent of elongation, and the rate at which they progress

through the cell cycle (Berger *et al.*, 1998). Therefore the outgrowth of the root hair can be considered to be a late differentiation event.

Figure 1.4.3.2 Root Hair Patterning in *Arabidopsis*



A Schematic drawing of an *Arabidopsis* root in surface view in the root hair developmental zone. Files of epidermal cells consist entirely of root hair cells or hairless cells.

B Schematic drawing of a transverse section from the mature portion of an *Arabidopsis* seedling root. Root hairs are located in the clefts between adjacent cortical cells. Only three root hairs are shown in this figure because the length of epidermal cells generally prevents all eight root hair projections from being visible in a single transverse section.

Figure taken from Schiefelbein *et al.*, (1997).

Specifying fate

The simple correlation between cell position and cell-type differentiation implies that lateral signalling events between cortical and epidermal cells help to define cell identity (Benfey and Scheifelbein, 1994), which is controlled by a cassette of transcriptional regulators. Interestingly, the genes that control cell fate within the epidermis of the root

also have functional equivalents that control the fate of cells within the epidermis of the shoot (Berger *et al.*, 1998; Benfey, 1999), however specification in the root epidermis is simpler as only two cell types exist, hairs and non-hairs, whilst stomata, trichomes and pavement cells have to be specified in the shoot. *WEREWOLF* (*WER*) and *GLABRA2* (*GL2*) are both expressed in non-hair cells and are required for non-hair cell fate, mutations in both result in hairs developing where non-hairs should be specified (Masucci *et al.*, 1996; Lee and Schiefelbein, 1999). *WER* encodes a MYB-related transcription factor that is required for the expression of *GL2*, which encodes an HD-ZIP homeodomain protein (Rerie *et al.*, 1994; Lee and Schiefelbein, 1999).

Both are therefore negative regulators of root hair development. A second MYB-related gene is *CAPRICE* (*CPC*), which contains a single MYB repeat but lacks the transcription activation domain (Wada *et al.*, 1997). *cpc* mutants develop few root hairs and so it has been proposed to act as a positive regulator, however a new model for its action has emerged since the cloning of *WER*.

Models of lateral inhibition are not new in biological systems however they have not been shown to exist in plants until now. Lateral inhibition involves cell to cell interactions, such that the differentiation of one cell inhibits the neighbouring cells from following the same fate (Wigglesworth, 1940). In this situation, non-hair cells represent the default state and so would prevent adjacent cells from following this fate by causing the activation of the hair cell fate. Analysis of the interactions of *WER*, *CPC* and *GL2* through the use of mutants and reporter gene combinations has shown how this works in the *Arabidopsis* root (Lee and Schiefelbein, 2002). The expression pattern is established by the heart stage of embryogenesis (Lin and Schiefelbein, 2001), and the position-dependent expression of *WER* is the key to the correct expression of the downstream genes, and thus the pattern itself. *WER* up-regulates both *CPC* and *GL2* within non-hair cells. *CPC* then causes a down-regulation of *WER* and *GL2* expression within the neighbouring cell, inducing the hair cell fate to be followed. In this model the cellular ratio of *WER* and *CPC* determine cell fate, with high levels of both resulting in a complex that could induce *GL2* expression and so induce the non-hair fate. Low levels would therefore result in an inactive complex that would be unable to induce transcription, so generating a root hair cell (Lee and Schiefelbein, 1999). Positional information creates an increase in *WER* in the non-hair cell position, *CPC* then moves, possibly through a plasmodesmal connection into the cell in the hair fate position, where

the lack of *WER* causes an inhibition of the expression of *WER*, *GL2* and *CPC* itself, resulting in the hair cell fate being followed (Lee and Schiefelbein, 2002).

Root Hair Outgrowth

Hair cell development can be subdivided into stages: selection of a site for hair formation, swelling formation, transition to tip growth and tip growth proper (Dolan *et al.*, 1994). The first visible sign of root-hair formation is the development of the swelling, or bulge, at the apical end of the cell (the site of hair emergence). The polar localisation of the bulge requires *ROOT HAIR DEVELOPMENT6* (*RHD6*) (Masucci and Schiefelbein, 1994), and results in a localised increase in calcium ions that develops into a tip-high gradient as the hair elongates, and which the *rhd2* mutant fails to form (Wymer *et al.*, 1997).

Following hair emergence, the root hair elongates through the polarised deposition of the new cell wall at the tip, a process called tip growth, and which also occurs during pollen tube growth. The elongation of the root hair by tip growth requires both auxin and ethylene, and can accelerate to a growth rate of 1μm min^{-1} (Dolan, 2001). Mutations in a cellulose synthase encoding gene, *KOJAK* (*KJK*), cause root hairs to initiate but instead of elongating they burst, resulting the death of the cell (Favery *et al.*, 2001). The nature of the gene suggests that it directly affects the structural integrity of the wall and its ability to withstand the pressure of tip growth.

If the root hair is to achieve polarised and directed tip growth, it is essential that wall loosening enzymes and the components of the new cell wall are directed to the tip. Microtubules form longitudinal cables along the length of the root hair, and when this organisation is disrupted chemically by either a microtubule depolymerising drug (oryzalin), or with a microtubule stabilising drug (taxol), the direction of tip growth is disrupted (Bibikova *et al.*, 1999). Consistent with these pharmacological observations is the wavy and bulging root hair phenotype of the *microtubule organising1*(*mor1*) mutant (Whittington *et al.*, 2001). *MOR1* encodes a microtubule-associated protein, and may have a role in stabilising microtubule arrays. More recently, the cloning of the *ECTOPIC ROOT HAIR3* gene has provided firm evidence for a role for the microtubules in not only the development of the hair itself but also in cell fate specification early on in development (Webb *et al.*, 2002). *ERH3* encodes a katanin-p60 catalytic subunit, a protein that severs microtubules. Mutants develop hairs in the non-hair position.

F-actin microfilaments accumulate at the site of the bulge and then at the tip of the growing hair, along which they are also arranged longitudinally down the length of the hair (Baluska *et al.*, 2000). Disruption of microtubules alters the direction of growth, but chemical disruption of the microfilaments causes a complete halt in growth, suggesting that they are probably involved in delivering vesicles to the tip (Dolan, 2001). Actin microfilaments are organised by a group of proteins called actin depolymerisation factors (*ADF*s), which bind to actin and regulate the polymerisation of microfilaments. The expression of some members of this family, such as *ADF3*, has been localised in the developing bulge of both maize and *Arabidopsis* root hairs (Jiang *et al.*, 1997; Dong *et al.*, 2001), implying an early role in reorganisation to allow the polar localised outgrowth of the root hair.

Recently a group of small *RHO*-related GTPases from plants (*ROP*) have been implicated in regulating the cytoskeleton during all stages of root hair development, from the initial localisation of the bulge to the tip growth in the elongating root hair (Molendijk *et al.*, 2001; Jones *et al.*, 2002). *RHO* GTPases are ubiquitous eukaryotic molecular switches that have already well documented roles in development, and form part of the *RAS* superfamily of monomeric GTPases. *ROP*s have been shown to be involved in the growth of pollen tubes, which, along with root hairs, are the only plant cells to undergo tip growth. *ROP1* directs the polar growth of the pollen tube through establishing the tip high calcium gradient and organising the actin cytoskeleton at the tip (Li *et al.*, 1999; Fu *et al.*, 2001). However apart from the *ROP*s known to be involved in pollen tube growth, the majority of the 11 *ROP*s identified in *Arabidopsis* had not been investigated.

Constitutively activated (CA) versions of *ROP4* and *ROP6* caused abnormal root hair growth, and *ROP4/6::GFP* protein fusions localised to the bulge and then to the tip of the growing root hair in wild type seedlings (Molendijk *et al.*, 2001). However, it appears that *ROP4* and *ROP6* may not normally be expressed in root hairs, as *ROP2* is the only one so far identified in a cDNA library made from root hairs (Jones *et al.*, 2002). *ROP2* therefore represents a root hair specific *ROP*.

Expression of *CA-ROP2* in wild type seedlings caused multiple root hairs to initiate from each cell and to develop branched tips, a response reminiscent of when plants are grown in the presence of chemical inhibitors of microtubules (Bibikova *et al.*, 1999).

Conversely, dominant negative (DN, i.e. GDP-bound) *ROP2* expressing plants had reduced root hair initiation sites and short wavy root hairs. Also, expression of a GFP-mouse talin marker within the lines expressing the recombinant *ROP2* proteins (CA and DN) was found to be abnormal, implicating an interaction with the F-actin cytoskeleton at the tip of the root hair. *ROP2* therefore controls both the frequency and the position of the bulge, as well as the amount of tip growth, which is equivalent to the role of *ROP1* in the growth of pollen tubes. Consistent with a role in these processes, a *ROP2::GFP* protein fusion was localised to the future site of root hair formation before evidence of swelling, and then to the tip of the elongating root hair for the remainder of its development (Jones *et al.*, 2002). This expression pattern matched that of *ROP*-specific antibodies reported by Molendijk and co-authors (2001). Taken together, these observations indicate that the *ROP2* GTPase acts as a positive regulatory switch in the earliest visible stages of root hair development, from the formation of the bulge through to tip growth of the hair itself.

It is therefore possible that *ROP2* interacts with both the actin microfilaments, through ADF proteins, and the microtubule network in order to bring about the correct localisation of the bulge and the normal directed tip growth of the emerging hair. Through this interaction the delivery of vesicles containing either calcium, cell wall loosening enzymes, or new cell wall material is directed towards the tip of the root hair. However it is important to note that this is purely speculation, and further work is required to shed light upon these possible interactions.

Auxin and Ethylene Promote Root Hair Outgrowth

A number of early chemical experiments showed that ACC was given to seedlings, root hairs developed in ectopic positions, indicating a key regulatory role for ethylene (Masucci and Schiefelbein, 1994; Tanimoto *et al.*, 1995). Consistent with this, seedlings incubated with silver ions or AVG showed reduced amounts of root hair initiation and growth. Mutants in the signalling pathway itself also exhibited abnormal root hair development. The *constitutive triple response1* (*ctr1*) mutant has constitutive ethylene responses and showed a hairier phenotype (Dolan *et al.*, 1994), whilst the ethylene overproducing mutant *eto1-1* exhibited much longer root hairs (Pitts *et al.*, 1998). Comparatively the ethylene insensitive mutants *ein2* and *etr1* both developed shorter root hairs however they showed no significant effect on root hair initiation (Masucci and Schiefelbein, 1996; Pitts *et al.*, 1998). Together these results implicate a role for

ethylene in determining the position of the root hair cells, as well as the outgrowth of the hair itself.

Auxin has a more specific effect on root hair length, as both the *axr1-12* and *aux1-7* mutations showed little effect on root hair initiation, producing a wild type density of shorter root hairs (Masucci and Schiefelbein, 1996; Pitts *et al.*, 1998). So both auxin and ethylene are involved in increasing the length of the root hair, however only ethylene induces ectopic root hairs to develop. By using a *GL2::GUS* reporter construct, Masucci and Schiefelbein showed that *GL2* expression was unaffected within the *axr2* and *ctr1* mutant backgrounds, and that the exogenous supply of auxin or ethylene did not alter the position specific expression of the reporter (Masucci and Schiefelbein, 1996). They suggest that both hormones act later in the developmental pathway, once the cell has entered the elongation zone.

The expression of the *AXR3* protein in wild type seedlings is restricted to elongation zone, however in the *axr3-1* mutants the protein is seven times more stable and so its influence may not be restricted to this zone (Ouellet *et al.*, 2001). *axr3-1* mutants have bald, hair-less roots, indicating that *AXR3*, and therefore auxin, is required for the development of root hairs, however as well as being auxin resistant they also show some ethylene resistance (Leyser *et al.*, 1996; K. Knox and O. Leyser, York University, personal communication). When the *axr3-1* mutant protein is expressed under a heat-inducible promoter fused to its own promoter, the part of the root that was in the zone of elongation and differentiation at the time of the heat-shock induction, does not produce any root hairs (K. Knox and O. Leyser, York University, personal communication). A preliminary model proposes that the normal *AXR3* protein is degraded by the time the cell enters the zone of differentiation, however as the *axr3-1* protein is degraded seven-time slower, it still represses auxin-induced gene expression as the cell starts to undergo root hair production within the differentiation zone.

1.4.4 Project Aims and Objectives

The overall aim of this work was to further our understanding of the genetic and hormonal control of aspects of plant development through the study of embryonic and seedling defective mutants. Specifically, the objectives of the work described in this thesis were to carry out a phenotypic and genetic characterisation of two *Arabidopsis* mutants defective in morphogenesis, in order to gain an insight into the possible nature of the roles of the disrupted genes.

Two recessive seedling-lethal mutants defined by two loci were identified in a population of T-DNA mutants of *Arabidopsis*, and designated *hydra1* and *hydra2* (Topping *et al.*, 1997; Souter *et al.*, 2002). The *hydra1* phenotype was initially outlined in the first *hydra* paper by Topping *et al.*, (1997), however a number of interesting traits had not been picked up, and so a closer inspection of this mutant was required. The main focus of this work though, was *hydra2*, which had not been previously described, phenotypically or genetically, and because of the similarity of the *hydra1* and *hydra2* phenotypes, and subsequently the nature of the disrupted genes, both were examined in parallel.

1.4.5 Summary

This chapter has highlighted the importance of signalling in orchestrating both embryonic and post-embryonic development, with particular relevance placed upon the use of *Arabidopsis* as an experimental species. The advantage gained through the work of the *Arabidopsis* Genome Initiative, and the application of different molecular genetic approaches to the study of signalling in development have been described.

Subsequent chapters will describe the methods used (Chapter 2) and the results of the genetic, phenotypic and preliminary molecular characterisation of the two mutants under study (Chapters 3 to 6). Chapter 7 will discuss the results obtained and assess their biological significance.

Chapter 2

Materials and Methods

Introduction

Described in this chapter are the materials and methods used to obtain the results described in the succeeding results chapters.

2.1 Materials

2.1.1 Chemicals

All chemical reagents were analytical reagent grade and were obtained from Sigma (Poole, UK), Fisher Scientific (Loughborough, UK), and BDH (Lutterworth, UK), unless otherwise stated.

2.1.2 Plant lines

In a screen of T-DNA and EMS mutants of *Arabidopsis*, a class of recessive seedling-lethal mutants defined by two loci were identified and designated *hydra1* and *hydra2* (Topping *et al.*, 1997). *Arabidopsis thaliana* ecotypes C24 and Wassilewskia (WS), along with the *hydra1* and *hydra2* lines were supplied by Prof. Keith Lindsey (University of Durham).

2.2 Plant Growth Conditions

2.2.1 Plants Germinated on Soil

For bulking of seed, crossing experiments and embryogenesis studies, plants were germinated on soil. Soil was made up to a mixture of 4:1 Levington's Multipurpose Compost to silver sand (to enhance drainage). Soil and sand were obtained from Klondyke Garden Centre, Chester-le-Street, UK.

Soil, once mixed, was autoclaved before potting so as to ensure that any unwanted seeds or fly larvae that might already be present in the soil would be destroyed. Seeds were then sprinkled onto autoclaved soil before covering with aluminium foil (to ensure uniform germination), and incubated at 4°C for one week; the *hydra* mutants required at least one week's vernalisation. After vernalisation, pots were moved to the greenhouse for germination. Seven days after germination, young seedlings were then transferred to individual pots containing non-autoclaved soil (autoclaving destroys soil nutrients).

Aracon tubes (BetaTech, Belgium) were used to aid collection of seeds, and were placed onto the plants once the first inflorescence was produced.

Pest Control Measures

Soil was initially watered with Intercept™ (Levington, UK) at a concentration of 64mg/tray (each tray contained 24 pots). Intercept™ is an insecticide that contains 70% (w/w) imidacloprid that is used as a one-off soil drench. Intercept™ was used to control sciarid fly, greenfly, whitefly and vine weevil. Biocontrol of thrips was also used in the growth rooms, through the use of mites (novartis.com).

Greenhouse Conditions

The green house was at a temperature of 22 ± 3°C, with a light regime of 16 hours light to 8 hours dark. Plants were sub-irrigated.

2.2.2 Plants Germinated on Culture Media Under Sterile Conditions

Culture Media

For sterile culture of plant material, half-strength Murashige and Skoog medium was used; 2.2g/l Murashige and Skoog basal mix, 10g/l sucrose and 3.25g/l of Phytagel at pH 5.8 (1M KOH). Phytagel was used to make all media, except when antibiotics were added, when Bactoagar was used. Agar was autoclaved at 121°C for 20 minutes before use, and then poured under sterile conditions in a sterile air flow cabinet.

Seed Sterilisation, Plating-out and Germination

Seeds were sterilised and washed in 70% ethanol (30 seconds), 50% hyperchlorite bleach (15 minutes), and autoclaved distilled water (three washes). Sterile seeds were then transferred to 100mm^2 sterile Petri dishes (Fred Baker) containing media with sterile disposable pipettes. Plates were labelled and then sealed with 3M Micropore™ surgical tape (Health Care, USA). To ensure uniform germination, plates were wrapped in aluminium foil before placing into the cold store (at 4°C) for one week to vernalize.

Sterile growth room conditions

The tissue culture growth room was at a temperature of 25°C, with 16 hours light/day, at a photon flux density of 50-150μmol/m^2/s.

Transfer of seedlings under sterile conditions

Seeds were allowed to germinate on horizontal plates. Seedlings were then transferred to fresh plates five days after germination. Secondary plates were then incubated vertically, so as to avoid the problem of the seedlings reaching the bottom of the horizontal plates. Transferring seedlings ensured that plates were not crowded. Heterozygous *HYDRA/hydra* lines were used in all experiments because both the *hydra* mutants are homozygous lethal.

Seedlings were transferred onto normal agar plates as well as plates supplemented with growth promoters and inhibitors. Transfers were undertaken in a sterile Flow hood, with sterile conditions maximised through the ethanol washing and subsequent flaming of forceps.

2.3 Physiological Response Experiments

2.3.1 Root Growth

Quantification of root growth in the mutants was required in order to assess the impact of different mutations and conditions on the mutant seedlings.

Primary Root Length Assays

Because of the more predictable growth of the main root when compared with the lateral and anchor roots, the primary root was chosen to study in all root growth experiments. The length of the primary root was measured every three days. The distance from the root tip to the root-hypocotyl junction was measured under a dissecting microscope. The same plants were used for all measurements with each developmental time-course.

Statistical Analysis of Primary Root Lengths

Root measurements were processed using a Casio fx-911v series calculator. The mean and standard deviation of the root measurements taken for each day was determined. The standard deviation was used to calculate the standard error of the mean, to give a value of the mean ± the standard error of the mean.

2.3.2 Gravitropic Responses

In order to identify mutant lines with agravitropic roots, such as *aux1* and *eir1*, it was necessary to undertake gravity response experiments.

Seedlings were germinated and then transferred to fresh vertical plates 3 days after germination as described above, ensuring that all roots were aligned. Once aligned, the plate was turned 90°. This was called time point zero or the point of gravistimulation. Seedlings were photographed from the same position every 30 minutes for a total of 180 minutes. In the case of the *hydra* responses, photographs were taken after 12, 24, 36 and 48 hours depending on when the response had been completed.

2.4 Exogenous Hormone Response Experiments

Exogenous hormone application presents a quick although crude way of altering internal hormone levels; the amounts used are often higher than physiological levels. However, this method was used to screen for altered responses in the mutants, so that further, more precise experiments (such as genetic manipulation) could be designed.

Hormones and Signalling Inhibitors

Seedlings were transferred as described above to agar plates supplemented with either a hormone or an inhibitor of hormone action (see list below for chemicals used). Hormones and signalling inhibitor compounds were made up in their respective solvent at stock concentrations, then filter-sterilised through 0.2μm pore Acrodiscs™ (Gelman, Northampton, UK), before use. The respective hormone or inhibitor was added to molten autoclaved media before pouring the plate; autoclaving destroys the activity of the chemical.

Hormones and inhibitors used in experiments:

Auxins

2,4-D	Synthetic auxin analogue. Enters the cell via the influx carrier.
NAA	Auxin analogue. Enters the cell by diffusion, and does not require the influx carrier.

Auxin transport inhibitors (phytotropins)

NPA	A synthetic auxin transport inhibitor. Inhibits efflux carrier.

NOA A synthetic auxin transport inhibitor. Inhibits influx carrier.

Brassinosteroids

Epibrassinolide (22R, 23R, 24R-2, 3-2a, 3a, 22, 23-tetrahydroxy-B-homo-7-oxa-5a-ergostan-6-one)

Cytokinin

BA

Ethylene

ACC Rate-limiting ethylene precursor.

Ethylene inhibitors

AVG Inhibitor of ACC synthase, the rate-limiting enzyme of ethylene biosynthesis.

$AgNO_3$ Silver ions inhibit ethylene perception at the receptor.

Hormone responses were quantified through root growth measurements as described above.

2.5 Analysis of Genetic Interactions Through the Creation of Double Mutants by Crossing

In order to investigate the genetic interactions involved in the establishment of the *hydra* phenotype, heterozygous *HYDRA/hydra* plants were used to cross with hormone mutants defective in hormone synthesis or perception as well as with GUS and GFP marker lines.

For a complete list of all mutant, GUS and GFP lines used in these experiments, see Appendix 1.

Embryo Selection of Heterozygous *HYDRA/hydra* Plants

Homozygous *hydra* mutants are infertile and do not grow on soil (Topping *et al.*, 1997). Therefore, populations of *hydra* plants were "typed" using embryo selection to isolate

heterozygous plants (*HYDRA/hydra*). Four late-stage siliques from each individual were screened for the presence of mutant embryos. A fine needle was used to slice open the silique before mounting the embryos onto a slide in water. A cover slip was used to squash the embryo out of the testa. Homozygous wild types were discarded, and heterozygous plants were kept for crossing.

Emasculation and Pollination

Plants were firstly assessed for suitable flowers, buds that were too young or already dehiscing pollen were discarded. Well developed, non-self-pollinated flowers were used as these proved to be the most successful. All siliques and buds not used for crossing were cut off of the stem being used. The most important of the buds to be removed were the young buds not being used at the apex.

Fine watchmaker forceps were used to emasculate (remove the anthers from) the flowers under a stereomicroscope. The top of the anther was removed so as not to damage the filament and the rest of the flower too much. Pollen was transferred from the male parent plant using fine watchmakers forceps, whole anther and filament being taken to ensure pollen transfer during "rubbing" of the dehiscing anther with the recipient stigma of the female parent. The stem was marked with a ring of 3M Micropore™ surgical tape (Health Care, USA).

Silique Development and F1 Seed Collection

Successful crosses developed a silique after three days, and any stigmata not developing were given a second dusting of fresh pollen. The F1 seed was collected when the silique had turned yellow, just before shattering. Seeds were dried out before being germinated on autoclaved soil.

Collection and Screening of the F2 Generation

The F1 generation were allowed to self and set seed. Each F1 parent was harvested individually so that non-*hydra*-positive plants could be discarded. Seeds were germinated under sterile conditions and screened firstly for the presence of the *hydra* mutation, and secondly for the presence of the second mutant/marker line.

The first screening criterion was the presence of *hydra* mutants within the line. Once these were seen, a second criterion was examined. In the case of a GUS marker or a

GFP marker line, the wild-type looking plants were removed and tested for GUS or GFP expression. However if the cross involved two mutants, then an extra phenotypic test was carried out. Details of all selection criteria used are mentioned individually in their respective results sections. Once a line containing both the *hydra* mutation and the second mutation was found, the line was subjected to further analysis.

Statistical Analysis of Double Mutants

To ensure that the double mutants were present, which was not required in the case of GUS or GFP marker lines, statistical analysis was required to show the segregation ratios of the two mutations.

Because *hydra* is a homozygous lethal mutation, no homozygous lines could be used at any stage. Heterozygous lines produced 3 wild type to every 1 mutant seedling. Within the population of mutant seedlings, which accounted for 4 out of 16, only 1 was homozygous for both mutations. In all experiments *hydra* mutants were moved to fresh plates and then the segregation of the phenotypes within this populations was determined.

Phenotypic Analysis of Double Mutants

Details of the phenotypic analysis of each mutant line is mentioned in the respective results chapter. See GUS and GFP analysis for details of how expression analysis was carried out.

2.6 Analysis of GUS Marker Lines

GUS marker lines offer an insight into hormonal and genetic interactions within a different background without the need for many complex experiments. However, interpretation of these expression patterns requires a thorough understanding of the original marker line being used. In order to meet this need, all lines were first subjected to an extensive developmental staining analysis.

Optimal Staining Time

The optimal staining time was determined in a time-course experiment. Lines were stained for different lengths of time, starting at 30 minutes and extending to several

hours. At each time point the staining was analysed using light microscopy. Table 2.6.1 below shows the GUS lines used and the optimal staining time for each line.

Table 2.6.1 Optimal GUS Staining Times

GUS Line	Staining Time
ACC SYNTHASE1	8 hours
CYCAT CYCLIN B1	3 hours
DR5	1 hour
EXORDIUM	2 hours
IAA2	1.5 hours
LIPID TRANSFER PROTEIN1	12 hours
PIN-FORMED1	6 hours
VASCULAR TISSUE1	24 hours

2.6.1 Histochemical GUS Staining

Tissue localisation of GUS enzyme activity was determined by staining at 37°C in 1mM 5-bromo-4-chloro-3-inodyl–D-glucuronic acid (X-gluc; Melford Laboratories, Suffolk, UK. Jefferson *et al.*, 1987). The buffer used was modified, comprising 100mM sodium phosphate (pH 7.0), 10mM EDTA, 0.1% Triton X-100 (Stomp, 1990), and 1mM potassium ferri/ferrocyanide to inhibit diffusion of the reaction intermediate.

2.6.2 Microscopic Analysis of GUS Marker Lines

Once stained, lines were cleared in 80% ethanol for 24 hours, with regular changes of clean ethanol. Once cleared, seedlings were then treated for microscope analysis as described in Section 2.8.2.

2.7 Analysis of GFP Marker Lines

Some of the markers lines used were GFP lines rather than GUS lines. The benefits of GFP are that precise localisation is obtained when only a small number of cells are expressing the reporter, and that staining can be seen without killing the plant, which is

what happens when histochemical localisation of GUS occurs. This method was taken from Wysocka-Diller *et al.*, (2000).

Tissue was counterstained with 10μg/ml propidium iodide and mounted in water. GFP fluorescence was visualised in whole mounts using a confocal laser scanning microscope (Leica). The FITC channel (green: GFP) was overlaid onto the TRITC channel (red: autofluorescence and propidium iodide) to permit identification of the GFP-expressing cells.

2.8 Microscopic Analysis of *Arabidopsis* Through Development

Light microscopy was used for many different experiments. In order to understand the precise patterning of different tissues within the mutants, and the expression patterns of different markers, light microscopy was essential.

2.8.1 Whole Mount Embryo Analysis

This method was adapted from Diener *et al.*, (2000). Siliques were collected so that all developmental stages were represented, but with particular interest in the very young siliques so that the early stages of embryogenesis could be seen. The siliques were then top-and-tailed and then fixed overnight in an ethanol : acetic acid (9:1) solution. After fixing they were rinsed in 90% and then 70% ethanol before being cleared in a solution of chloral hydrate : glycerol : water (8:1:2) (v/v), for 1 hour at room temperature.

Because of the toxicity of chloral hydrate gloves were worn and fine tweezers were used to carefully open the silique and remove the embryos, being careful not to damage them. Embryos were then mounted on slides in a 40% glycerol solution, and viewed with DIC optics under a light microscope.

2.8.2 Whole Mount Seedling Analysis Using DIC Optics

This method has been adapted from Malamy and Benfey, (1997). Seedlings were cleared in 80% ethanol overnight, with frequent changes of clean 80% ethanol. Tissue was then incubated at 57°C in a 0.24M HCl 20% methanol solution for 15 minutes, and then transferred to a 7% NaOH 60% ethanol solution for 15 minutes at room temperature. Seedlings were re-hydrated through an ethanol series of 40%, 20% and 10% ethanol (five minute incubations in each), then infiltrated in 5% ethanol / 25% glycerol for five minutes. Seedlings were then mounted in 50% glycerol and viewed using DIC optics. GUS stained, lugol stained, and cleared tissue was viewed in this way.

2.8.3 Lugol Staining

In order to view the root cap columella lugol solution was used to stain the starch granules within columella cells. Seedlings were stained for 3 minutes in a 10% lugol, 25% glycerol solution inside a dark box (as lugol photobleaches), and then washed in fresh 50% glycerol before mounting and viewing immediately under a light microscope with DIC optics. Images were captured quickly so as to avoid photobleaching.

2.8.4 Whole Plant Visualisation of the Vascular Network

In order to understand the elaboration of the vascular network in the mutants, and to assess continuity and the interconnections of the vascular system, this method from Mattsson *et al.*, (1999) was used. Seedlings were incubated in chloral hydrate (8g), water (2ml), and glycerol (1ml) for one hour at room temperature, and then mounted in glycerol on slides and viewed under dark-field optics.

2.8.5 Tissue Maceration for Analysis of Vascular Elements Using DIC Optics

In order to isolate and view in detail the vascular elements within the vascular tissues, whole tissues were incubated at 80°C in an equal volume solution of glacial acetic acid and hydrogen peroxide (20 volume), for 6 hours. Hydrogen peroxide strength is very important as if it is too strong, then the mixture can become explosive. Tissue was then put in 5 ml of water in a universal tube, and then shaken very hard, resulting in a

suspension of cells. This was then centrifuged down to produce a more concentrated mass of cells. Cells were then mounted in glycerol and covered with a cover slip, and then viewed under DIC optics.

2.8.6 Preparation of Material for Histological Analysis

For a thorough histological analysis of the mutants, material was embedded and then sectioned for light microscope work. Material was firstly fixed, dehydrated, and then embedded in LR White embedding resin (London Resin Company, UK).

Tissue Fixation and Dehydration:

Tissue was fixed under vacuum, in 4% paraformaldehyde and 2.5% gluteraldehyde, in a 1M phosphate buffer solution. Samples were then washed with 1M phosphate buffer three times before being taken through a dehydration series of dry ethanol; 30, 50, 70, 95 and 100% ethanol.

Infiltration and Embedding:

LR White resin was infiltrated into the samples through a graded series of LR White : ethanol; 1:2, 1:1, 2:1, and pure LR White. Once in pure LR White, samples were oriented in 8 mm flat bottomed BEEM polypropylene capsules (BEEM, UK), and filled with LR White so as to exclude all air when the capsule was closed. Polymerisation occurred at 70°C.

Sectioning and Staining:

Semi-thin sections for light microscopy were cut on a Bright rotary retracting microtome (Bright Instrument Co. Ltd., Huntingdon, UK). Sections were cut using glass knifes and then floated onto a water bath and then transferred to electrostatically charged slides (Superfrost Plus, BDH, Lutterworth, UK), for improved binding.

Tissue was stained with Toludine Blue (a general tissue stain, lignin staining blue-green), before being sealed permanently with a cover slip (BDH, UK), using DPX Mounting medium (Fisher Scientific, UK). Samples were then viewed under a light microscope.

2.8.7 Wax Embedded Serial Sectioning of the Hypocotyl Region

In order to fully investigate the patterning of different tissue types throughout the hypocotyl, a systematic analysis was undertaken by sectioning through the hypocotyl. Wax embedded hypocotyl sections were preferred because the process of sectioning wax embedded material produced a continuous ribbon of sections, allowing a straight-forward sequential reconstruction of the tissue to be undertaken.

Acridine Orange was used to stain and view the sections taken and was chosen because it fluoresces, with excitation at 488nm being detected with an emission filter at 530nm. Sections were therefore viewed using a Leica confocal microscope, which was preferable to a normal fluorescent microscope because the laser allowed the collection of images focused on a 1μm plane, despite using 8μm sections. 8μm sections were taken because this allowed greater tissue preservation and cleaner sectioning of the wax embedded material. As a result of these considerations, the following method was employed.

Fixation

Seven day old seedlings were fixed overnight in methanol / acetic acid (3:1) fixative at -20°C, then washed three times in PBS for 15 minutes each.

Dehydration and Embedding of Material in Paraffin Wax

Seedlings were dehydrated through an ethanol series; 10, 30, 50, 70, 90% for 15 minutes each, followed by two 45 minute washes in 96% and 100% ethanol.

Infiltration with Histoclear was as follows:

EtOH / Histoclear 3 : 1, 60 minutes.

EtOH / Histoclear 1 : 1, 60 minutes.

EtOH / Histoclear 1 : 3, 60 minutes.

100% Histoclear, 60 minutes.

100% Histoclear, overnight

Following an overnight incubation in pure Histoclear, a layer of melted Paraplast (Sigma) paraffin wax (at 60°C) was poured over the top of the Histoclear containing the specimen, and incubated at room temperature for 12 hours. A second layer of melted paraffin (at 60°C) was then poured over the Histoclear/paraffin wax mix, and left for a further incubation at 42°C for 12 hours. After the addition of a third layer of melted paraffin (at 60°C), the mixture was incubated at 58°C for 4 hours. Half the

volume of the Histoclear/paraffin wax mixture was then replaced with pure melted paraffin and incubated at 58°C for 4 hours. After two hours another round of replacement of the Histoclear/paraffin mix was performed. Finally the whole volume of Histoclear/paraffin mix was replaced with pure melted paraffin, and incubated at 58°C overnight. The volume of paraffin was then replaced with pure melted paraffin a further three times over the following 12 hours whilst incubating at 58°C.

Metal frame moulds were prepared and then pure paraffin wax (at 60°C) was poured into one mould at a time. Immediately after the wax had been poured and a skin formed on the side walls of the mould, the tissue was transferred to the mould using pre-heated tweezers, so as to reduce the amount of wax setting, along with the sample, on the tweezers. The tissue was oriented correctly before a skin was blown over the top of the mould, so as to aid setting and reduce movement of the sample. A label was added to the setting block, showing the number and type of tissue embedded, as well as its orientation. The whole mould and setting block were then placed in cold water to aid fast setting of the wax.

Sectioning

8μm sections were cut using a disposable knife (Accu-Edge®, Raymond A Lamb, London), that was regularly cleaned using 70% ethanol, and replaced when worn. Ribbons of sections were collected, cut into segments of four to five sections (depending on how many would fit across the slide), floated on a heated de-ionised water bath (at 40-45°C) and then collected on polyL-Lysin coated slides (BDH). The chronological order was maintained during collection on slides. Slides were labelled and numbered, and then incubated on a 37°C hot plate over night to stick the sections to the slides.

Deparaffinisation and Re-Hydration

Deparaffinisation was undertaken as follows, through a Histoclear and ethanol series:

20 min. in 100% Histoclear
10 min. in 100% Ethanol
10 min. in 95% Ethanol
10 min. in 70% Ethanol
10 min. in dH_2O

Staining and Viewing

Slides were stained in a 10% aqueous solution of Acradine Orange for 2-5 minutes then washed in water for 1 minute. Slides were drained of as much water as possible, without allowing it to dry completely, before one drop of Slowfade® Light Anti-fade reagent in glycerol (S-7461, Molecular Probes, Leiden, The Netherlands) was added to the slide before being covered with a Type 1 thickness coverslip (BDH). An anti-fade reagent was used because a fluorescent stain will diminish quickly in the light. The anti-fade reagent reduces such diminishing of the signal by up to 60% (Molecular Probes, Leiden, The Netherlands). Sections were viewed using a Leica confocal microscope, excitation at 488nm being detected with an emission filter at 530nm. An image was captured from every third section so as to give a good indication of the sequence of the tissue pattern.

2.8.8 Scanning Electron Microscope (SEM) Analysis

In order to analyse the epidermal surface of both the shoot and the root of the *hydra* mutants, in particular the development of root hairs, SEM analysis was undertaken.

Plants were initially grown in soft-set agar, but this caused problems with the agar sticking to the root hairs and not allowing a good image to be taken. Liquid media was then tried, however this increased the water-stress response of the plant, a response mediated by ethylene – a hormone that promotes root hair growth, and so this was discarded. Roots were therefore grown on the surface of hard-set agar.

Preparation of Samples

Seedlings were grown on vertical plates so that the roots did not grow into the agar, then carefully peeled off of the agar for fixation.

Fixation and Dehydration

Seedlings were fixed under vacuum for 30 minutes in a 4% paraformaldehyde solution made with 0.1M phosphate buffer. The vacuum was broken two to three times during the 30 minute incubation to encourage the infiltration of the fixative. After fixation the samples were washed several times in 0.1M phosphate buffer.

Specimens were then dehydrated through an ethanol series: 30, 50, 70, 95 and 100%, incubating in each percentage for a minimum of 3 hour. Slow dehydration gave the best preservation of the root hair and epidermal cell structure.

Critical Point Drying

Once fully dehydrated samples were dried under liquid carbon dioxide. Carbon dioxide lyophilises, or changes from a liquid to a gaseous phase instantaneously at a specific temperature and pressure, at the critical point.

Samples were placed in 100% acetone for 10 minutes before placing inside the home-made drying machine. The acetone was then replaced with liquid carbon dioxide under high pressure. A water jacket was used to heat-up the temperature of the chamber towards the critical point, when the carbon dioxide lyophilised.

Dried samples were mounted onto metal stubs (Agar Scientific, Stanstead, UK) (12.5mm diameter), covered with adhesive carbon discs (Agar Scientific, Stanstead, UK).

Sputter Coating and Viewing

Samples were coated with an electron-dense layer of gold-palladium of 5-50 Å, using a Polaron sputter coating machine.

A JEOL IC848 Scanning Electron Microscope was used to view the samples, images were digitally captured, before processing using Adobe Photoshop 5.

2.9 Analysis of PIN Protein Expression in the Root

In order to investigate the localisation of the auxin efflux carrier proteins within the root, indirect immunolocalisation of PIN proteins was undertaken. This work was performed in collaboration with Jiri Friml in Dr Klaus Palme's laboratory at the Max-Planck-Institut für Züchtungsforchung, Köln, Germany.

Solutions

Microtubule Stabilisation Buffer (MTSB): 50mM piperazine ethanesulfonic acid (PIPES), 5mM ethylene glycol tetraacetic acid (EGTA), 5mM $MgSO_4$, H_2O, pH 6.9-7.0 using KOH.

Fixation and Staining

Tissue was fixed in 4% paraformaldehyde in MTSB under vacuum for 1 hour, with the vacuum being broken three times in order to increase infiltration of the fixative.

Tissue was then permeabilized, washed and stained as follows using an *Insitu* Pro robot (Intavis) programmed to carry out the incubations. Five washes with Triton X100 0.1% in MTSB were followed by five washes with Triton X100 0.1% in sdH_2O, and then the tissue was permeabilized by incubating with 2% Driselase in MTSB for 30 minutes at room temperature. Samples were again washed five times with Triton X100 0.1% in MTSB, before incubating with a solution of 10% DMSO, 0.5% Nonident P40 in MTSB for 1 hour, changing for fresh after 30 minutes, and then washing five times with Triton X100 0.1% in MTSB. Blocking was done by incubating for 1 hour in 3% BSA in MTSB, before incubating for 4 hours with *PIN*-specific primary antibody diluted (1:40) in 3% BSA / MTSB. Samples were again washed five times with Triton X100 0.1% in MTSB and then stained for 3 hours with FITC conjugated anti-rabbit secondary antibody (Dianova) (1:2000) diluted in 3% BSA / MTSB. Two final sets of washes were then carried out; five washes with Triton X100 0.1% in MTSB was followed by five washes with sdH_2O.

Antibodies

Specific PIN antibodies were previously generated using a recombinant protein and then affinity purified (Muller *et al.*, 1998; Galweiler *et al.*, 1998; Friml *et al.*, 2002b).

Mounting and Viewing

After the staining procedure the roots were exceptionally delicate, so they were floated onto water baths in order to ensure that they could be untangled without loss of the root tip. Fine tweezers were used to mount the roots onto slides, before mounting in Slowfade® Light Anti-fade reagent in glycerol (S-7461, Molecular Probes, Leiden, The Netherlands).

In order to ensure that the roots were flat on the slide, and that the distance between the cover-slip and the slide was as minimal as possible, the aerial parts of the plant were often carefully removed, as well as reducing the amount of Slowfade® mount. This ensures that the laser is able to focus the whole of the root in one plane.

Samples were viewed on a confocal microscope (Leica DMIRBE, TCS 4D), with fluorescein isothiocyanate (FITC) specific detection at 530 ± 15nm, and auto-florescence specific detection at 580 ± 15nm. Images were captured and exported to Adobe Photoshop 4.0, where the two images were overlaid, resulting in red auto-florescence and green-yellow FITC specific fluorescence.

2.10 Analysis of the *hydra2* Gene

In order to quickly and confidently identify determine the gene which had been disrupted in the *hydra2* line, a series of experiments were carried out.

2.10.1 RNA Extraction for RT-PCR Analysis

In order to determine if an RNA transcript for the *HYDRA2/FACKEL* gene was produced in the *hydra2* line, RT-PCR was used to specifically amplify the *FACKEL* RNA transcript. For this reaction RNA was collected from *hydra2* mutants and WS wild type seedlings.

The Qiagen RNeasy extraction kit and method were used for this procedure, as it is a quick and simple method, and did not require a large amount of starting material.

Procedure

Mutants and wild type plants were harvested from sterile plates and placed in Eppendorf tubes that had a hole pierced in their lids. Each sample was then dropped into liquid nitrogen, the hole in the Eppendorf ensured that the tube did not explode when it rapidly cooled. Samples were stored at -80°C until required.

A pestle and mortar were baked overnight at 240°C so as to reduce contamination and DNase and RNase activity. The pestle and mortar were then cooled with liquid nitrogen before the sample was added. Samples were kept on liquid nitrogen until required. A fresh pestle and mortar were used for each sample.

The sample was added to the pestle along with 450µl of RLT lysis buffer and some more liquid nitrogen to grind up the tissue before it could thaw. RLT lysis buffer stock

solution was made by mixing 990µl RLT Buffer with 10µl of β-mercarptoethanol. RLT Buffer contains guanidine isothiocyanate (GITC), which gives it superior cell disruption abilities.

Once the sample had been ground completely a pre-chilled clean spatula was used to transfer the sample to an Eppendorf, which was left to thaw on ice.

After thawing, the lysate was transferred to a QIAshredder spin column in a 2ml collection tube, and centrifuged for 2 minutes at maximum speed (15,000 rpm) in a bench-top centrifuge. The flow-through was transferred to a fresh Eppendorf without disturbing the cell-debris pellet in the collection tube, and 225µl of ethanol (96-100%) were added and mixed by pipetting, and then transferred to an RNeasy mini spin column sat in a 2ml collection tube and centrifuged for 15 seconds at 10,000 rpm (8000g) in a bench-top centrifuge.

700µl of RW1 Buffer were pipetted onto the RNeasy mini spin column and centrifuged for 15 seconds at 10,000 rpm to wash. The flow-through and the collection tube were discarded, and the RNeasy column transferred to a new 2ml collection tube. 500µl of RPE Buffer were added onto the RNeasy column and centrifuged for 15 seconds at 10,000 rpm.

The flow-through was discarded, and then 500µl of RPE Buffer were added the column, which was centrifuged for 2 minutes at maximum speed. The flow-through and collection tube were again discarded. The RNeasy column was transferred to a new 1.5ml collection tube before 30µl of RNase-free water was added directly onto the membrane of the column. After allowing to sit for 1 minute the column was centrifuged for 1 minute at 10,000 rpm to elute the RNA sample, which was then stored at –80°C until required.

2.10.2 Quantification of RNA and DNA by Agarose Gel Electrophoresis

RNA and DNA can be separated, purified and quantified by electrophoresis through an agarose gel matrix. The range of size of RNA/DNA molecules separated can be altered by altering the agarose concentration. The migration of linear nucleic acid molecules

through the gel is directly proportional to the length of the molecule and so, by loading markers of known length alongside samples, it was possible to determine their size.

The position of the RNA/DNA within the gel following electrophoresis was visualised through the addition of ethidium bromide to the gel. This dye intercalates between bases of RNA/DNA. Dye-RNA/DNA complexes can be visualised due to their florescence under UV light.

A gel tray was taped up along the two open ends, ready for pouring. A 1% agarose in 1x TAE solution was heated in a microwave for 1 minute, mixed and then allowed to cool whilst 10µl of ethidium bromide were added per 100ml of molten agarose. The molten agarose was then poured into the gel tray, a comb added roughly 1-2cm from the end of the tray, and 2-3mm from the base of the tray, and then left to set for 20 minutes.

RNA/DNA samples were mixed with load dye (and sdH_2O if required) on a piece of Parafilm. 6x strength load dye was used at a final concentration of 1x. A fresh pipette tip was used for each sample so as to ensure there was no contamination of samples. Samples were loaded carefully into each well.

5µl of Hyperladder I (bioline.com) were run in the lane adjacent to samples. Hyperladder I contains 14 regularly spaced bands, ranging from 20bp to 10,000bp. Each band contains an exact amount and size of DNA fragments, so that the distance the band has travelled down the gel (determined by the size of the fragment) and the intensity of the band (determined by the amount of DNA/RNA) can be determined.

The gel tank was filled with 1x TAE, the gel aligned correctly and then run at 100V until completed. Following electrophoresis the RNA/DNA was visualised on a UV trans-illuminator and the image captured using the Molecular Analyst® software package (Bio-Rad, Hemel Hempstead, UK).

2.10.3 Quantification of RNA by Spectrophotometry

RNA (and DNA) can be quantified visually on an agarose gel, however in order to gain a more specific value, a spectrophotometer was also used.

40μg/ml RNA gives an absorbance of 1 at 260nm. The ratio of the reading for 260nm and 280nm can therefore be used to give an indication of the quality of the RNA sample. A ratio of 1.7 to >2 is expected for good quality RNA.

2μl of RNA were diluted in 198μl RNase-free water, and mixed thoroughly. Absorbance was measured at 260nm and 280nm, using water as a blank.
The amount of RNA was calculated using the following equation:

A_{260} x dilution factor (here 100) x 40 = μg/ml RNA

2.10.4 RT-PCR Analysis of the *HYDRA2/FACKEL* Gene in the *hydra2* Mutant.

This technique can be used to determine the presence or absence of a transcript, to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library. The system used for this experiment was the Promega Access RT-PCR kit. This is designed to do the reverse transcription (RT) and polymerase chain reaction (PCR) amplification in one tube, thereby simplifying the procedure and reducing the potential for contamination.

Reverse Transcriptase from *Avian Myeloblastosis Virus* (AMV) was used for first strand DNA synthesis. This enzyme can be used at 48°C, which reduces the potential for RNA secondary structures to form. Thermostable *Tfl* DNA Polymerase from *Thermus flavus* was used for second strand cDNA synthesis and DNA amplification. The polymerase chain reaction (PCR) results in the amplification of a segment of DNA between two regions of known sequence, catalysed by DNA Polymerase.
Reactions were carried out in a Perkins-Elmer thermal cycling machine.

Primers

Primer combinations suggested by Kathryn Schrick (University of Tuebingen) were used for the amplification of the entire coding region of the gene, and were made by Chruachem Ltd.

Primers used for RT-PCR of the *FACKEL* cDNA:

FK Forward 5'- ACA AAG CGA GAA AAG GCG ATA CAA ACG - 3'
FK Nested Forward 5'- ACG ATT TCG AAT TCT TCA TCT TCT CCT TTG -3'

FK Reverse 5'- GTA CTA CAA AGT TTC ACT CGA GCA TTG CTA -3'
FK Nested Reverse 5'- GGA TTC ATG ACT TGG CTC TTG GAC -3'

For details of the reaction mixture, see Appendix 2.

The reaction was initiated by adding the RNA template, and then a few drops of mineral oil were put over the reaction mixture so that it would not evaporate during the reaction.

Reaction Details

1 cycle at 48°C for 45 minutes for first strand cDNA synthesis by reverse transcription, was followed by 1 cycle at 94°C for 2 minutes to allow AMV RT inactivation and RNA/cDNA/primer denaturation.

Second strand cDNA synthesis and PCR amplification followed 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, and finally extension at 68°C for 2 minutes. A final 1 cycle extension at 68°C for 7 minutes was used to ensure that all extension had been completed. The PCR machine was then programmed to enter a 1 cycle soak to keep the samples at 4°C until they could be removed from the machine.
For details of the program used, see Appendix 2.

Analysis of 5% of the PCR products was done by agarose gel electrophoresis (as mentioned above, 2.10.2), and then reaction products were stored at -20°C until needed.

Actin Controls Used in all PCR Reactions

Actin primers were used as controls to ensure that PCR conditions were working correctly. Primers were designed such that two products could be detected. A single band at 550bp showed the reverse transcription of the RNA template. The appearance of a second band at 600bp represented genomic DNA, and therefore genomic contamination within the reaction mixture.

PCR analysis of RT-PCR products

In order to assess the success of the RT-PCR, a second round of PCR was performed using a set of nested primers designed to allow a second round of specific amplification, to reduce some of the background noise. A 1/10 and 1/100 dilution of the original

reaction mixture was taken, (experience later showed that the less template the more specific and efficient the amplification).

PCR Amplification

The reaction mixture contained BIOTAQ™ DNA Polymerase (bioline.com), NH_4 Reaction Buffer, $MgCl_2$, dNTPs, nuclease-free water, primers and reaction products.

Amplification followed 40 cycles of a standard programme, denaturation at 94°C for 30 seconds, annealing at 55-60°C for 1 minute, and extension at 68°C for 1.5 minutes. One final cycle of extension at 68°C for 7 minutes was used to ensure complete extension of all fragments. The PCR machine was then programmed to enter a 1 cycle soak to keep the samples at 4°C until they could be removed from the machine. See Appendix 2 for details of the program used.

Annealing temperature was varied depending on how specific the primer binding was required to be. Initially this was 55°C, but was soon raised to 65°C.
Extension time was calculated using the rough guide of 1 minute extension per kb of expected product, such that the 1kb expected product for *FACKEL* was given 1 minute extension time.

Chapter 3

Results:

Initial Characterisation of the *hydra* Mutants

Introduction

In a population of T-DNA mutants of *Arabidopsis*, a class of recessive seedling-lethal mutants defined by two loci were identified, and designated *hydra1* and *hydra2*. The aim of the work described in this first results chapter was to characterise for the first time the phenotype of *hydra2*, the primary subject of this work, as well as to describe in more detail the phenotype of *hydra1*; the *hydra1* phenotype was initially outlined in the first *hydra* paper by Topping *et al.*, (1997).

Both *hydra1* and *hydra2* share a number of phenotypic characteristics, and so it was decided that investigating the two mutants in parallel would be more informative than looking at just one of the mutants in isolation. To this end *hydra1* was re-investigated alongside *hydra2* throughout these studies. As a result of this more detailed analysis other characteristics were discovered that had not previously been identified.

3.1 Embryogenesis in the *hydra* Mutants is Disrupted From the Globular Stage Onwards

Topping *et al.*, (1997) reported abnormal embryo development in *hydra1*, and so in order to see if these problems were also seen in the *hydra2* line, characterisation of the mutant was started by looking at embryogenesis. Dissection of siliques allowed the analysis of developing embryos by light microscopy.

Consistent with the observations reported by Topping *et al.*, (1997), *hydra1* embryogenesis was disrupted from the early globular stage onwards (data not shown). Misoriented and mispositioned cell divisions throughout the axis created extra cell layers within all areas of the embryo. The transition to bilateral symmetry at the heart stage did not occur, indeed the upper region of the embryo had a number of developing cotyledon primordia. There were between 3 and 6 cotyledons normally visible, however they were typically not well developed, with reduced expansion, and are very often fused together. No distinct shoulder to the cotyledon primordia could be distinguished, the apical meristem disorganised and very often duplicated (Figure 3.1, H and I). Cell axialisation (elongation throughout the apical-basal axis) during the heart stage failed to occur within the central region, resulting in reduced growth of the embryonic hypocotyl and expansion of the cotyledons. During the later phases of embryogenesis the wild

Figure 3.1

Embryogenesis

A: Wild type embryo at the early globular stage (mag. x 200).

B: *hydra2* embryo at the early globular stage (mag. x 100).

C: *hydra2* embryo at the triangular stage (mag. x 100).

D: Wild type embryo at the heart stage (mag. x 100).

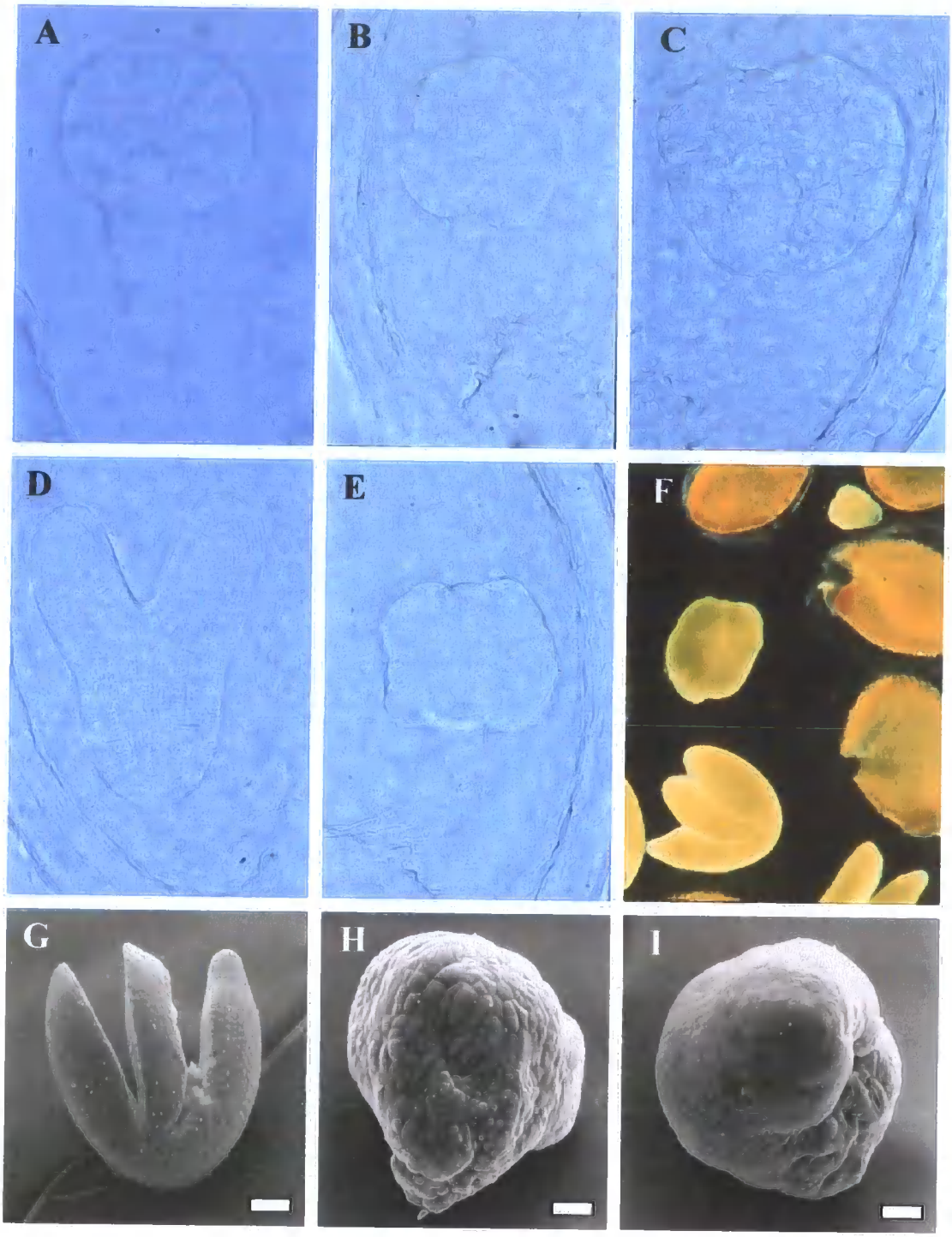
E: *hydra2* embryo at the heart stage (mag. x 100).

F: Mature wild type and *hydra2* embryos (mag. x 10).

G: Scanning electron micrograph of a mature wild type embryo (scale bar represents 200μm).

H and I: Scanning electron micrographs of mature *hydra1* embryo (scale bar represents 200μm).

Figure 3.1



type embryo formed the bent cotyledon stage (Figure 3.1, G), which was totally lacking within the *hydra1* mutants, indeed, since there was no elongation of the root tissues, the radially expanded upper and central regions of the embryo filled the seed (Figure 3.1, H and I). It appears that the hypophysis and suspensor develop normally (Topping *et al.*, 1997), and consistent with this observation is the fact that the root meristem is present.

hydra2 mutants mirrored this pattern of disruption, resulting in identical round mature embryos. Figure 3.1 shows the differences between a wild type embryo and a *hydra2* embryo. Normal development occurred up until the globular stage (Fig. 3.1, A and B), when random division planes created extra cells throughout the axis (Fig. 3.1, C). By the early heart stage multiple cotyledon primordia could be seen developing at the apex of the mutant embryo (Fig. 3.1, E), a difference that could be more clearly seen at later heart stage when the wild type has two cotyledons developing (Fig. 3.1, D). At this point cell axialisation throughout the hypocotyl and cotyledons should elongate the embryo, however the mutant embryos showed no elongation in any region, resulting in the round and squat embryo that matured by filling the seed rather than forming the bent cotyledon stage evident in the mature wild type embryo (Fig. 3.1, F). The wild type embryos possessed a root and hypocotyl, and two cotyledons bent over, whilst *hydra2* resembled a round ball. The root meristem was evident at the bottom of the *hydra2* embryo, and the cotyledons formed ridges on the top of the embryo.

3.2 *hydra* Seedling Growth and Development

A minimum of 7 days vernalisation at 4°C was required to ensure that all the *hydra* embryos germinated successfully. Because of the lack of an elongated embryonic axis the seed coat was not efficiently broken out of upon germination, often the seed coat was still covering the top of the seedling until it had grown enough to split it open and grow out of it. Therefore initially they resembled gnomes with hats on.

The *hydra* mutants could not be germinated on soil and so had to be germinated in sterile conditions on half-strength Murashige and Skoog medium. They were also unable to survive being transferred to soil even after germinating on sterile growth media.

A number of seedlings exhibited axis duplication to some extent, a defect that had occurred in embryogenesis and had created seedlings that appeared like Siamese twins; arrows indicate the presence of multinumerary organs Figure 3.2.1. This common characteristic was seen in both *hydra* mutants and had not been previously described. It occurred either throughout the whole axis at the same time, or was restricted to specific elements of it, such as double meristems in the shoot (Fig. 3.2.1, E and F) and/or in the root (Fig. 3.2.1, B and D). However the presence of two shoot meristems did not necessarily also have two root meristems. Double root meristems appeared to be correlated with the number of main vascular poles running through the entire length of the hypocotyl. If the numerous vascular poles joined together before entering the hypocotyl root junction then only one main root developed. However if more than one vascular strand entered the hypocotyl root junction, then a duplicated main root or more than one main root developed.

At 3 days old both *hydra1* and *hydra2* showed reduced root growth and reduced elongation of the hypocotyl, and by 6 days old there was a considerable difference in the root and aerial growth of the mutants when compared with wild type seedlings (Fig. 3.2.1, G). H shows this comparison at 9 days old, and I at 14 days old for comparison of the development of the shoot region. The wild type had elongated petioles of the leaves that formed the basis of a rosette, *hydra2* seedlings however had considerably reduced petioles and multinumerary compacted round leaves that created a cabbage-like growth habit.

The shoot region showed considerable disorganisation, a consequence of the extra shoot meristems and the multinumerary cotyledons that developed during embryogenesis. Wild type seedlings possess a simple phyllotaxis, developing two cotyledons, and then two first leaves opposite each other but in between the two cotyledons, which also face each other. From this pattern the next pair of leaves initiate at 137° to the previous pair, and so it continues creating the rosette in an ordered pattern. In the *hydras* the development of the cotyledons during embryogenesis was not organised or uniform, and consequently the position and the relative sizes of the first organs was not uniform. Figure 3.2.2 shows the position of the cotyledons and the subsequent positioning of the first leaf primordia in *hydra2*, which were misoriented in their position and disorganised in their axiality. In the wild type the leaves develop facing each other (adaxial side

Figure 3.2.1

***hydra* Development**

A: 3 day old *hydra2* seedlings.

B: 6 day old *hydra1* seedling.

C: 6 day old *hydra2* seedling.

D: 6 day old *hydra1* seedling.

E: 9 day old *hydra1* seedling.

F: 9 day old *hydra2* seedling.

G: WS and *hydra2* show growth comparisons, 6 DAG.

H: WS and *hydra2* show growth comparisons, 9 DAG.

I: WS and *hydra2* show growth comparisons, 15 DAG.

Scale bars represent 1mm.

Figure 3.2.1

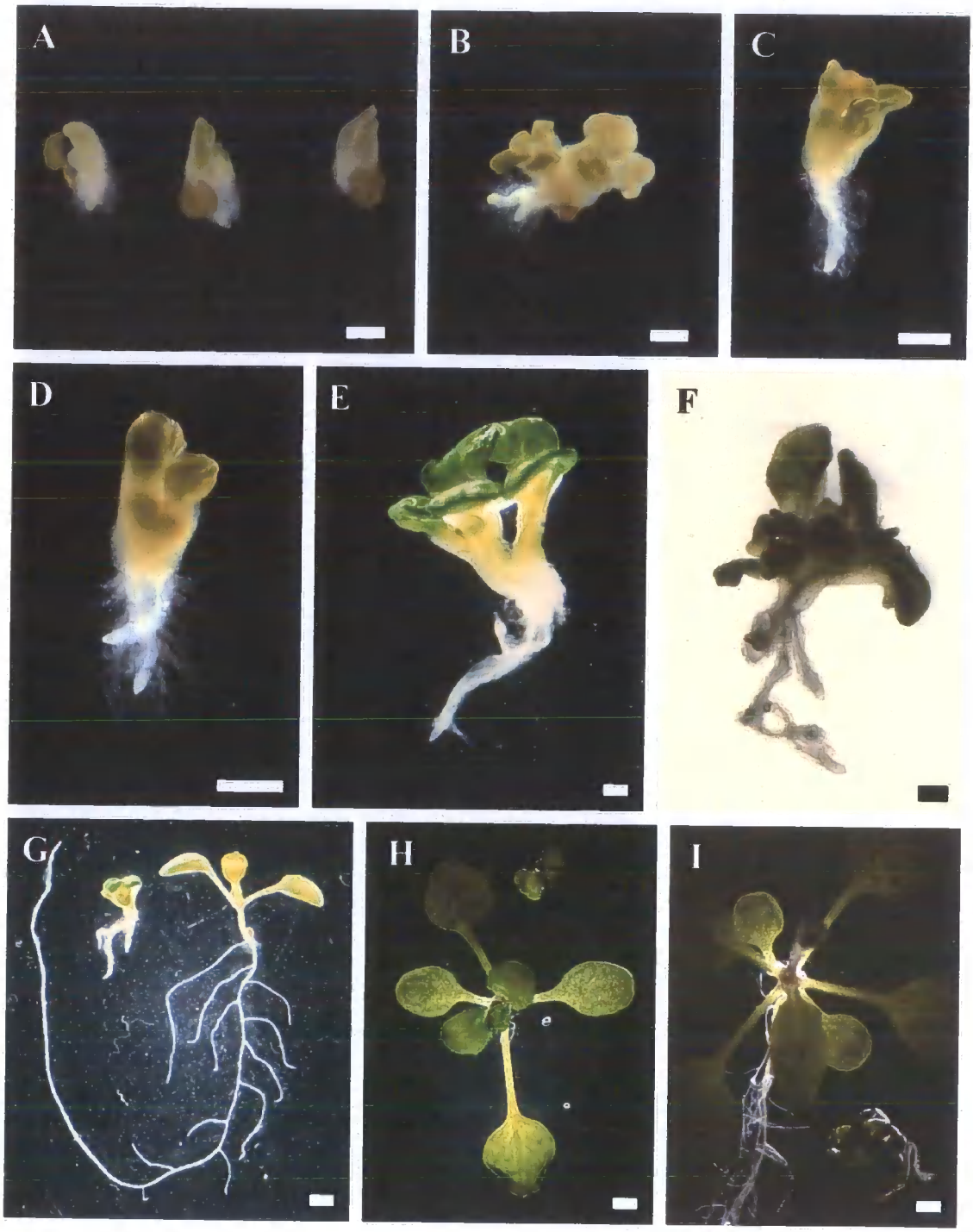


Figure 3.2.2

***hydra* Development**

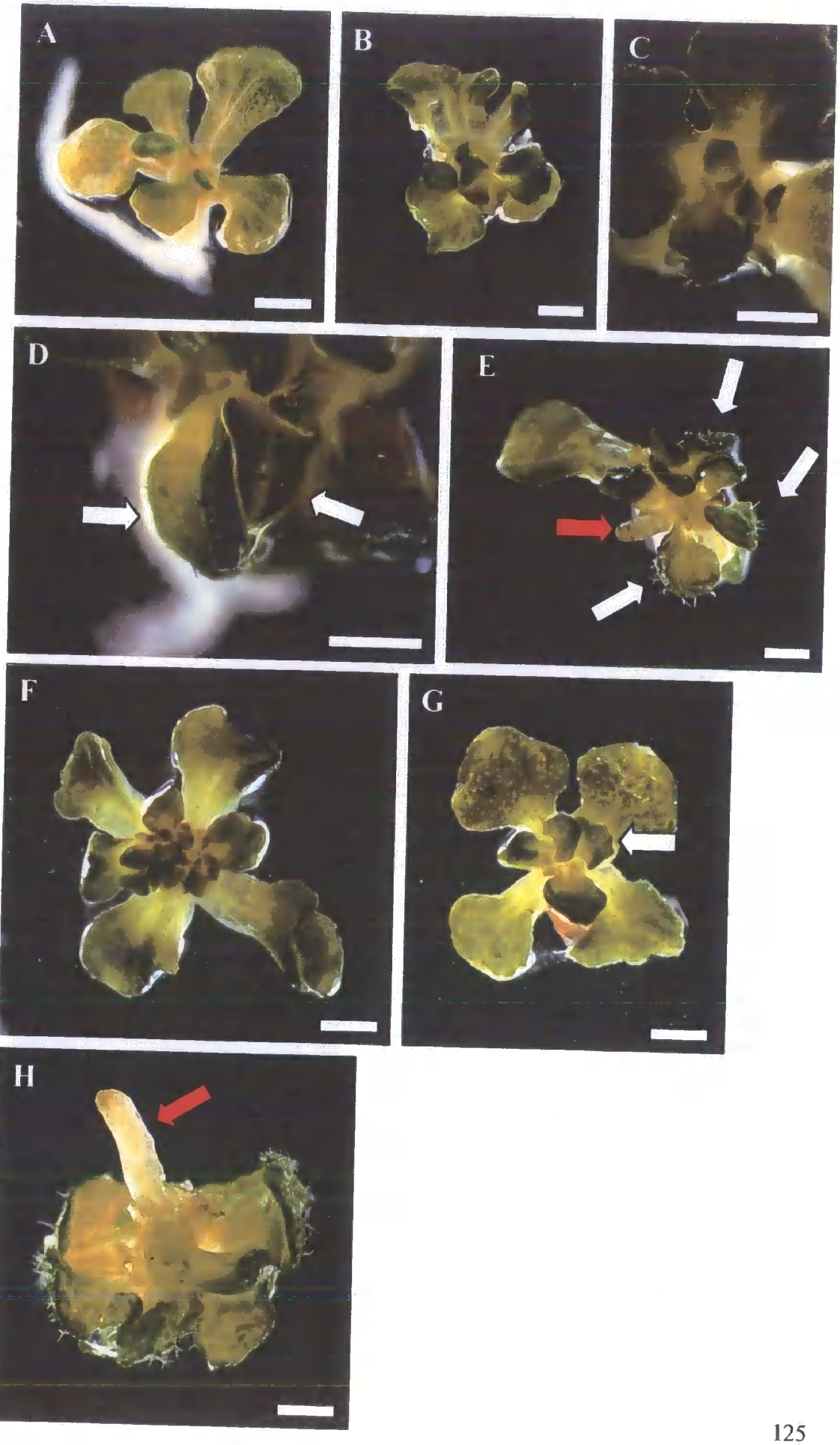
A, B, C and F show *hydra1*, 12 days after germination.

D, E, G and H show *hydra2*, 12 days after germination.

Scale bars represent 1mm.

Red arrows show pin-like cotyledons or leaves, and white arrows show confused orientation of the leaves, with trichomes on the leaf 'adaxial' surfaces.

Figure 3.2.2



facing inwards, and abaxial side facing outwards), however in the *hydras* they developed facing each other as well as facing back to back, and even facing front to back (travelling airline). To highlight this characteristic Figure 3.2.2, D, shows the abaxial-adaxial cell fate confusion by focusing on the trichomes, indicated by the white arrows. Trichomes form on inner-facing (adaxial) leaf surfaces, however because of the misorientation of the leaves, the *hydras* developed these adaxial features on the incorrect side of the leaves. The red arrows in Figure 3.2.2 E and H show pin-like cotyledon and leaf structures respectively.

3.3 Scanning Electron Microscope Analysis of the Shoot Region of *hydra* Mutants

Scanning Electron Microscope (SEM) analysis of the shoot region highlighted the observations made at the light microscope level. Figures 3.3a and b show the strict organisation and pattern that developed in the wild type shoot (Fig. 3.3a, A and B). Analysis of the shoot region of *hydra1* (Fig. 3.3a, C and F) and *hydra2* (Fig. 3.3a, D and E, and Fig. 3.3b, A, B and C) seedlings showed that the apical meristem was severely diverted from its normal developmental program. A number of leaf primordia were seen developing in a random pattern that showed no resemblance to the wild type phyllotaxis. The development of unevenly sized multinumerary cotyledons in an often non-symmetrical pattern, created mis-specified first leaf primordia to develop. The subsequent initiation of further primordia, that normally form in pairs directly opposite each other, was therefore irretrievably disrupted. The more random the position of the cotyledon themselves, the more the subsequent pattern was disrupted.

3.4 Increased *CYCAT1:CDB:GUS* Expression in the Shoot Meristem

The observations made of the shoot region in the *hydras* suggested that there may be a lack of correct patterning or an increase in the amount of cell division within the meristem. It was therefore important to be able to visualise in an indirect manner the cell division activity of the shoot meristem, as whole mount microscopical analysis was difficult due to the increased thickness of the *hydra* shoot region when compared with wild type. The *cyclin B CYCAT GUS* reporter line (Hauser and Bauer, 2000) provided a

Figure 3.3a

SEM Analysis of the Shoot Region of the *hydras*

A and B: Wild type 7 and 9 days old respectively.

C and F: *hydra1* 9 days old.

D and E: *hydra2* 9 days old.

Figure 3.3a

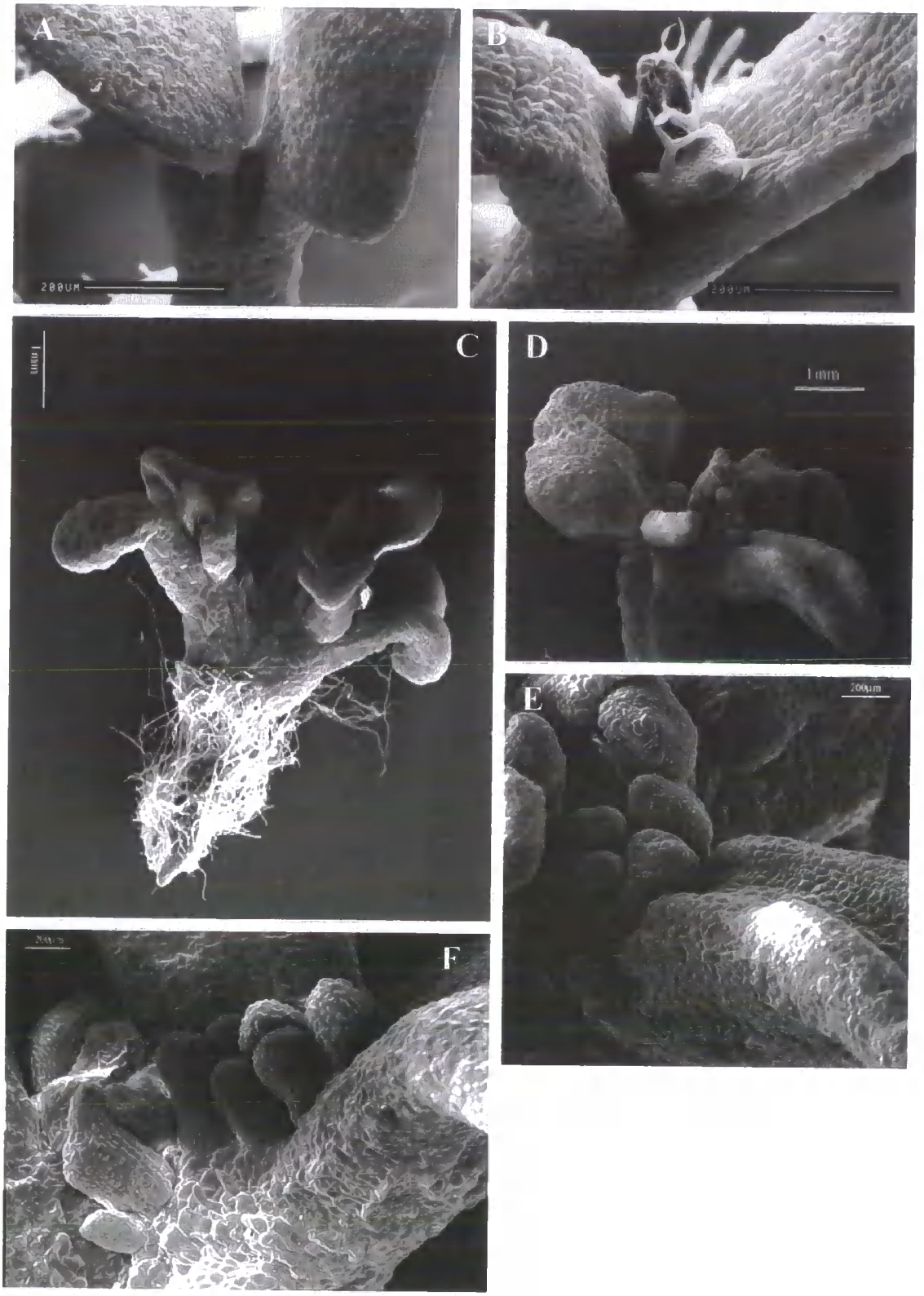


Figure 3.3b

SEM Analysis of the Shoot Region of the *hydras*

A, B and C: *hydra2* 12 days old.

Figure 3.3b

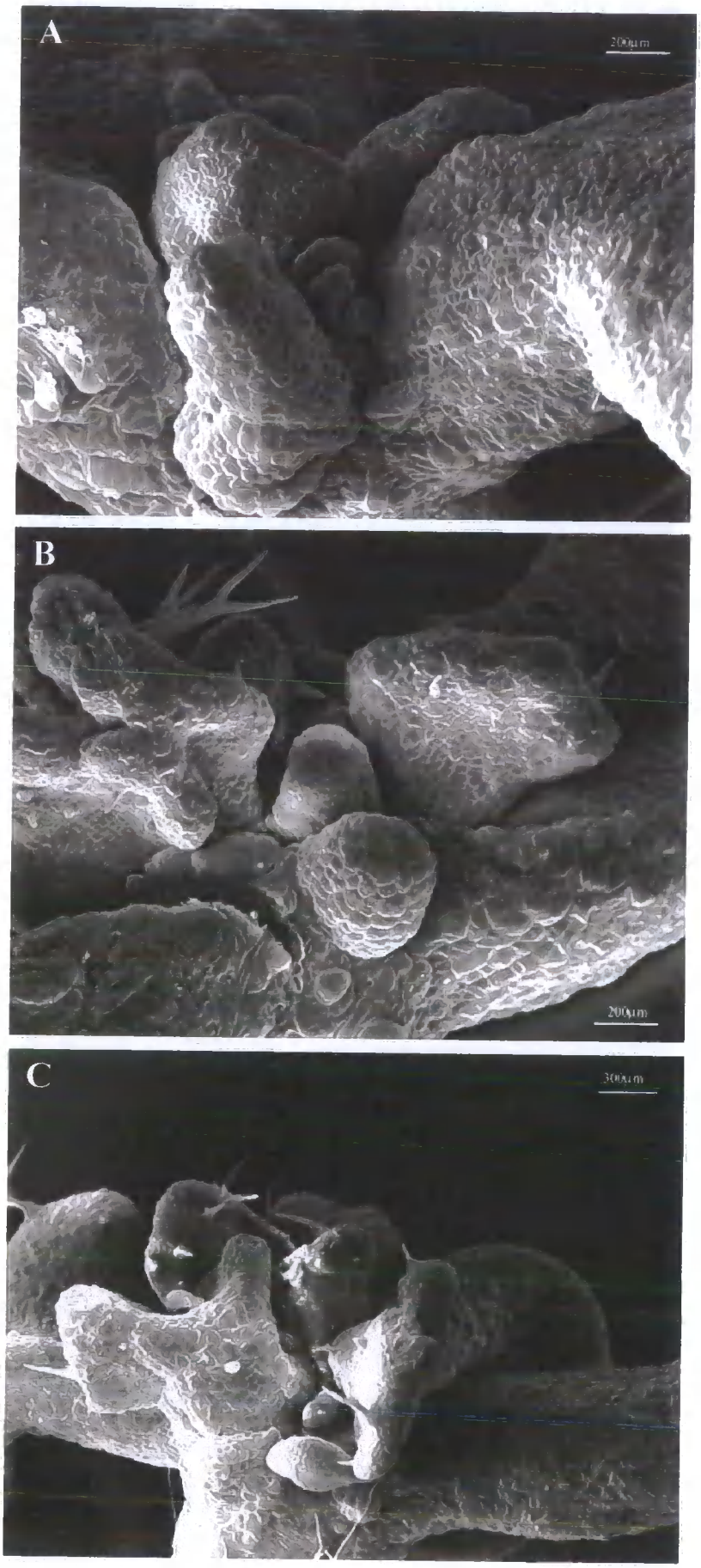


Figure 3.4

***CYCAT1:CDB:GUS* Expression in the *hydra2* Shoot**

A: Wild type *CYCAT* expression in 3 day old shoot meristem (mag. x 4).

B: Wild type *CYCAT* expression in 6 day old shoot meristem (mag. x 30).

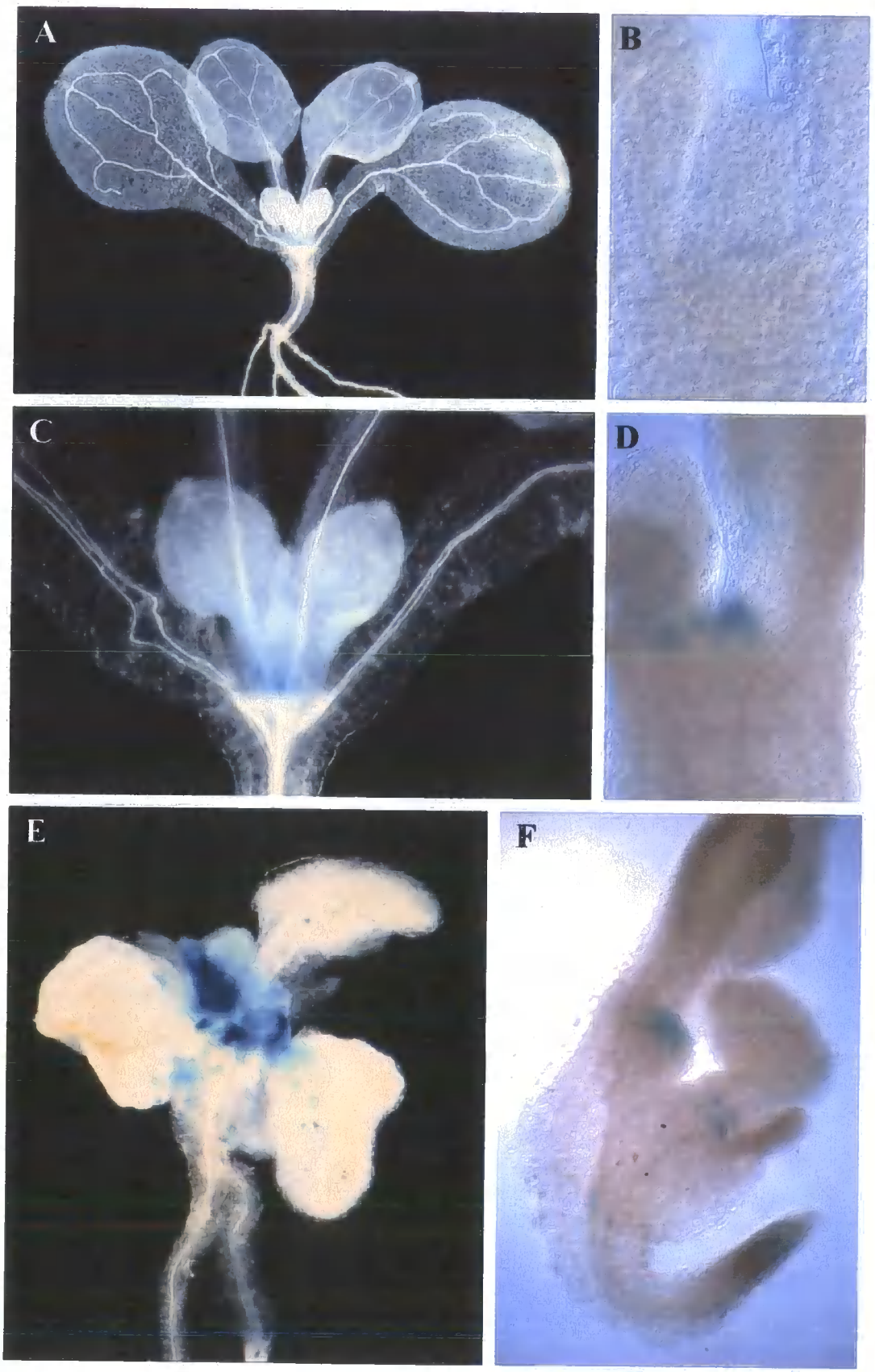
C: Wild type *CYCAT* expression in 9 day old shoot meristem (mag. x 10).

D: *CYCAT* expression in *hydra2*, 3 days after germination (mag. x 30).

E: *CYCAT* expression in *hydra2*, 9 days after germination (mag. x 6).

F: *CYCAT* expression in *hydra2*, 3 days after germination (mag. x 12).

Figure 3.4



method for visualising the level of cell division activity within the shoot meristem without the need for detailed microscopical analysis.

Cyclins are specifically synthesised and degraded at precise points in the cell cycle, and they achieve this because they contain a destruction box. The destruction box contains a conserved 9 amino acid signal sequence that recruits the proteasome machinery of the cell to degrade the protein. The *cyclin B CYCAT GUS* transgenic line was created in order to follow the cell cycle within the plant (Hauser and Bauer, 2000). The *cyclin B CYCAT* promoter was fused to the GUS reporter gene tagged with the Destruction Box signal sequence, so that the β-glucuronidase enzyme was destroyed at the same point in the cell cycle as the native cyclin B. The GUS stain is specific to cells that are in the process of dividing or have just divided, cyclin B accumulates in late G2 and is degraded by the end of mitosis (Hauser and Bauer, 2000).

Figure 3.4 shows that the expression pattern in the wild type was restricted to the base of the first leaf primordia (Fig. 3.4, A and C), with very little expression seen in the shoot three days post germination (Fig. 3.4, B). *hydra2* however showed an increase in the intensity and amount of staining 3 days post germination (Fig. 3.4, D), with duplicated staining patterns seen in seedlings that had undergone axis duplication (Fig. 3.4, F). By 9 days post germination the expression in *hydra2* had increased considerably (Fig. 3.4, E).

These observations clearly indicate that there is a large increase in the amount of cell division activity in the shoot meristem of the mutants. However this must be put in to context as the *hydra* shoot meristem is larger than the wild type, and so an increase in expression should be expected considering there are more cotyledons and therefore more first leaf primordia. If anything, then, these results highlight the increased size and activity of the *hydra* shoot meristem.

3.5 Death Before Flowering

As mentioned above, *hydra* mutants were never successfully transferred to soil. Only one *hydra1* plant has been seen to flower, however the flowers were male sterile

(Topping *et al.*, 1997). All *hydra* seedlings died after some 30 days grown in tissue culture, and no *hydra2* plants were seen to flower.

3.6 Vascular Patterning Throughout the Shoot of the *hydra* Mutants

The vascular pattern within the cotyledons and leaves of wild type plants is predictable and consistent, reflecting the size and shape of the organ. From the developing cotyledons in embryogenesis the subsequent differentiation of the main vascular strands through the hypocotyl ensures a continuous vascular system from the roots to the shoots. However the *hydras* have both supernumerary cotyledons and a radially swollen hypocotyl, suggesting that there would be abnormal patterning of the vascular tissue through the cotyledons, leaves and hypocotyl.

With this in mind it was essential to look at the vascular patterning within the abnormally shaped and sized cotyledons of the *hydra* mutants, and the subsequent patterning of the connections within the radially expanded hypocotyl. To do this tissue was cleared in chloral hydrate, and analysed in whole-mount so as to be able to visualise the connections throughout the system.

As Figure 3.6 shows, there was a considerable difference between the connections running through the wild type hypocotyl and cotyledons (Figure 3.6, A and D) and those running through the variably disrupted *hydra* mutants (Fig. 3.6, B, C, E, F, G and H). The vascular pattern within the cotyledons was often duplicated and nearly always disrupted, the degree of disruption depending on the type of cotyledon that had developed. Pin-like structures possessed one single vascular strand running through their length, whilst fused cotyledons had multiple main vascular strands entering through the petiole that caused a duplicated and fused pattern within the organ itself (Fig. 3.6, H). There were a number of vascular strands running through the hypocotyl region, connecting the shoot meristems and cotyledons with the roots (Fig. 3.6, E, F, G and H).

In order to assess the quality of the vascular tissue that was developing within the mutants, tissue was macerated and the vascular elements were then examined under high power on a light microscope. Fig. 3.6, I, shows the characteristically long and thin

Figure 3.6

Vascular Patterning Throughout the Shoot Region of the *hydra* Mutants

A: Wild type cotyledon, 9 DAG (mag. x 8).

B and C: *hydra1* cotyledons 9 DAG (B mag. x 10, C mag. x 6).

D: Wild type hypocotyl 9 DAG (mag. x 8).

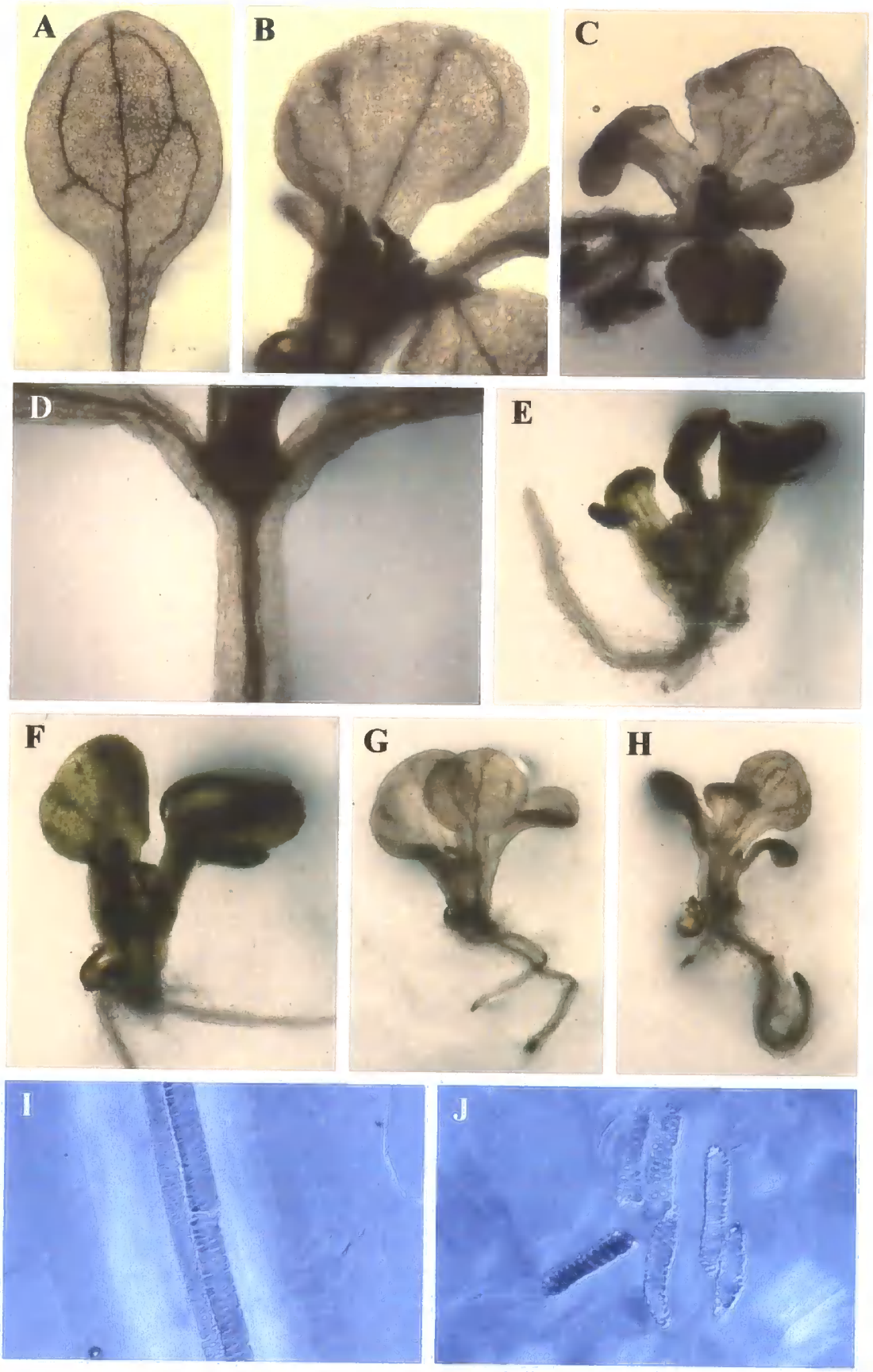
E: *hydra1* whole seedling 9 DAG (mag. x 6).

F, G and H: *hydra2* whole seedlings 9 DAG (mag. x 6).

I: Vascular elements from a wild type hypocotyl (mag. x 150).

J: Vascular elements from the hypocotyl of *hydra2* (mag. x 150).

Figure 3.6



vascular elements found in the wild type, with pitting along the lignified walls. The elements seen in Fig. 3.6, J, are those from *hydra2* and are at the same magnification as I. The mutants possessed short elements that although shorter in length were no different in width, and the pitting along the walls appeared normal.

These results indicate that the differentiation of the vascular elements within the disrupted cotyledons and hypocotyl region occurs normally, although they complete differentiation before they have elongated to their wild type length. Further investigation of the defects in vascular patterning were therefore required.

3.7 *VASCULAR TISSUE1* Expression in *hydra1*

One of the major problems with looking at the *hydra* mutants is that they are larger and thicker than wild type plants, and so visualisation techniques that work very well in wild type seedlings do not necessarily work as effectively with the mutants. So, in order to view the vascular patterning GUS reporter genes were crossed into the *hydra* background, such as the promoter-trap line *VASCULAR TISSUE1* (*VT1*) (Wei *et al.*, 1997). When *hydra1VT1* double mutants were studied, they showed the connections of the vascular tissue in the cotyledons with those in the hypocotyl very clearly.

The connections in the cotyledons correlated with those in the hypocotyl region, such that the position and number of cotyledons determined the extent and path of the vascular tissue running through the hypocotyl region (Figure 3.7, A-G). As reported earlier, there were often double vascular poles running through the hypocotyl.

3.8 *PIN-FORMED1::GUS* Expression in the Vascular Tissue

To further investigate the patterning of the vasculature throughout the plant a second GUS reporter line was also crossed into the *hydra* backgrounds. *PIN-FORMED1* promoter GUS (*PIN1::GUS*, from Klaus Palme) is a very strong staining GUS line when compared with *VT1*, which took 24 hours to achieve the same level of staining that was achieved in 6 hours with *PIN1::GUS*. This difference in strength had a major implication for the condition of the tissue after staining, which was more delicate after

Figure 3.7

VASCULAR TISSUE1* Expression in *hydra1

A: *VT1* expression in wild type 9 DAG (mag. x 8).

B to G: *VT1* expression in *hydra1* 5 DAG (B, E, F and G mag. x 8, C x 6, D x12).

Figure 3.7

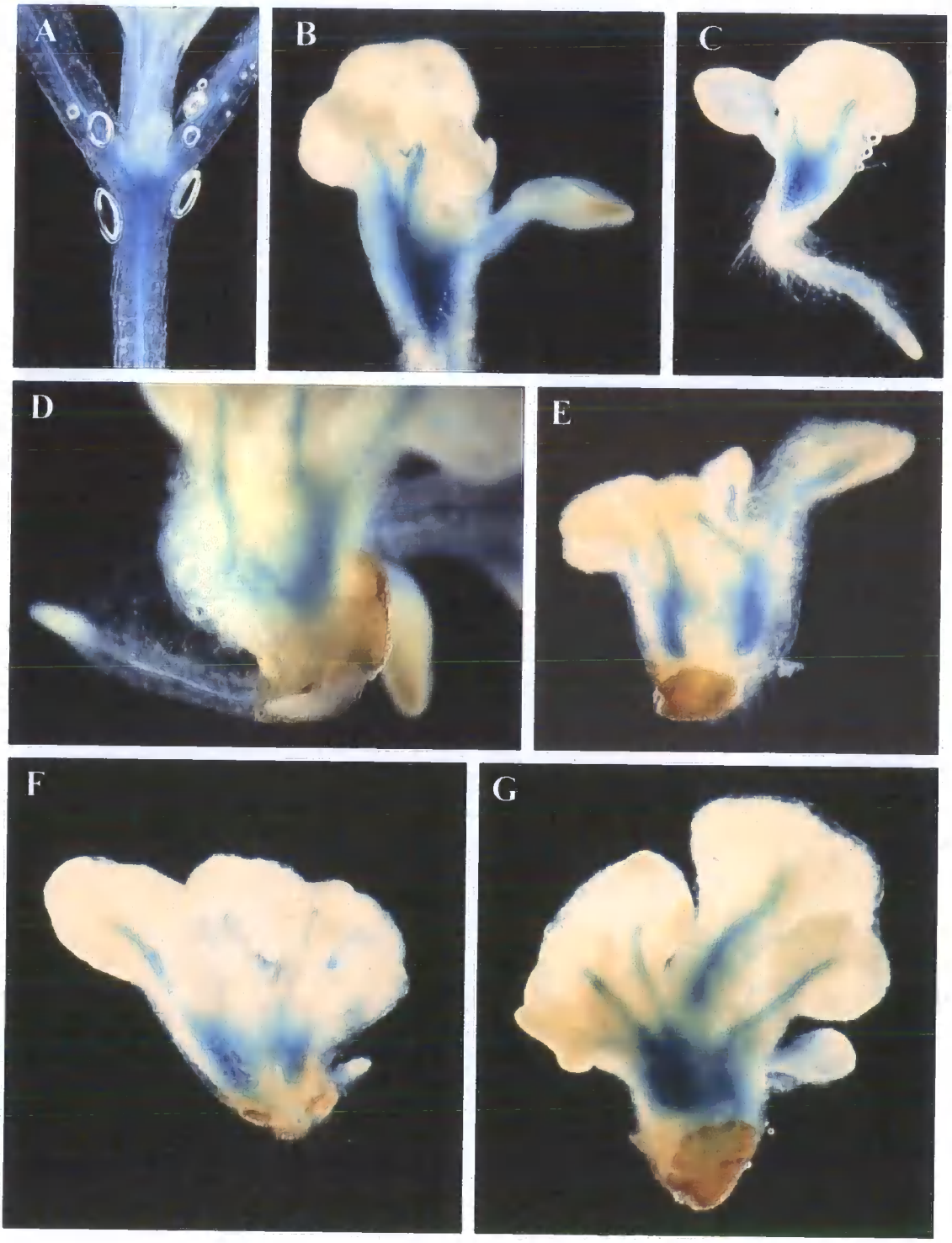


Figure 3.8

PIN1::GUS* Expression Pattern in *hydra2

A: Wild type *PIN1::GUS* expression in a leaf (mag. x 5).

B and C: *PIN1::GUS* expression in *hydra2* leaves (B mag. x 8, C mag. x 10).

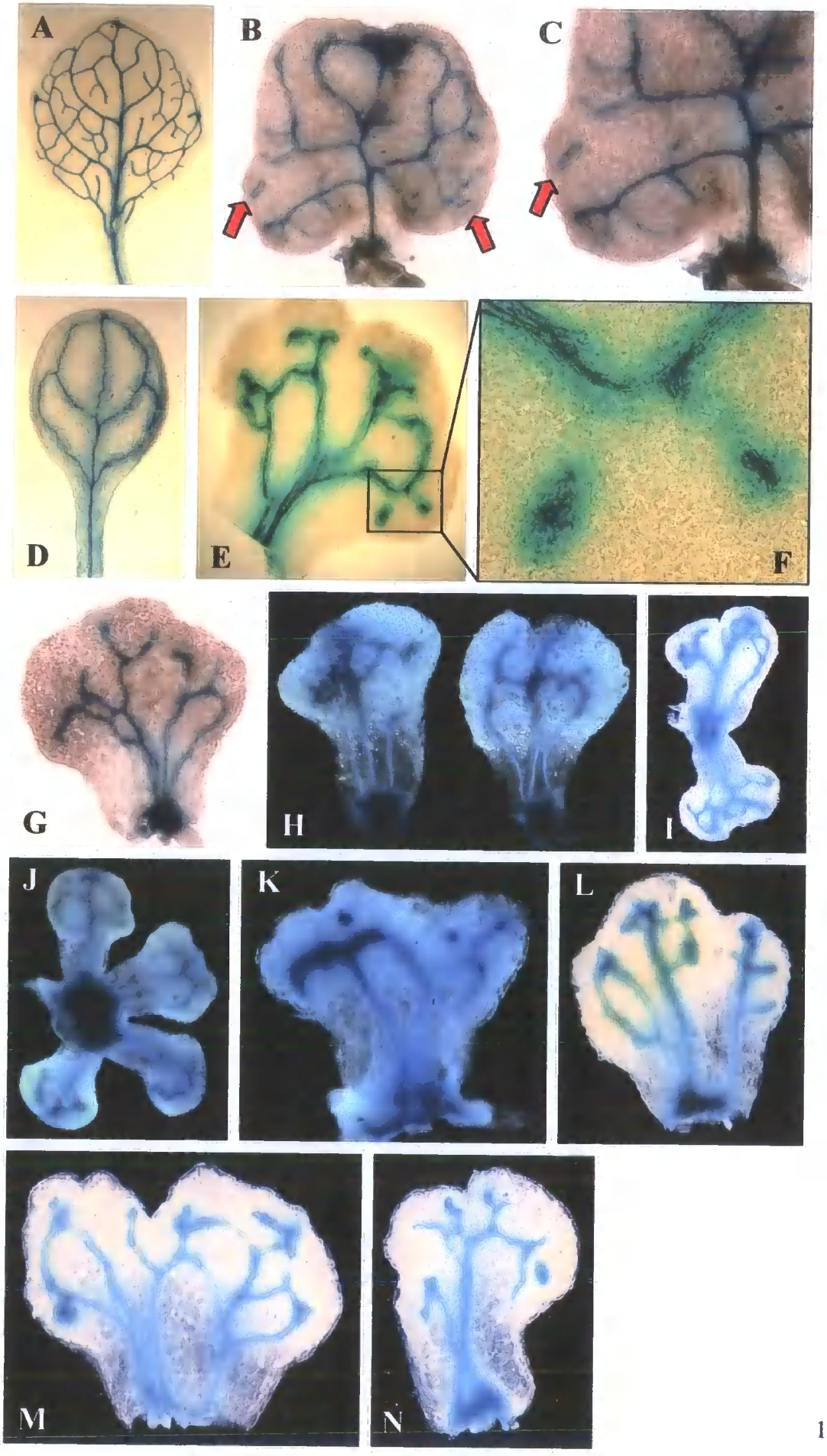
D: Wild type *PIN1::GUS* expression in a cotyledon (mag. x 6).

E to N: *PIN1::GUS* expression in *hydra2* cotyledons (E mag. x 10, F mag. x 40, G, H, K, L, M and N mag. x 10, I and J mag. x 5).

All seedlings 15 DAG.

Red arrows indicate isolated vascular elements.

Figure 3.8



24 hours in histochemical buffer at 37°C. Also, *PIN1::GUS* was found to stain the vascular tissue within the leaves and cotyledons better than the *VTI* line.

Figure 3.8 shows *PIN1::GUS* expression throughout the leaf vasculature in a wild type seedling (Fig. 3.8, A and D) and the *hydra2* mutant (Fig. 3.8, B, C and E to N). *PIN1::GUS* highlights the primary, secondary and tertiary veins in the wild type leaf (Fig. 3.8, A), as well as the predictable pattern in the wild type cotyledon (Fig. 3.8, D). The vascular patterning in *hydra2* leaves (Fig. 3.8, B and C) however shows isolated vascular elements that have differentiated but have not been 'plumbed-in' to the rest of the vasculature within that organ. The red arrows highlight these isolated vascular elements.

A combination of different vascular patterns were seen in *hydra2* cotyledons (Fig. 3.8, E to N), with isolated vascular elements also present (Fig. 3.8, E and F). F shows a higher magnification image demonstrating that *PIN1::GUS* marked differentiated vascular elements, and that isolated GUS staining correlated with the isolated vascular elements found in both the leaves and the cotyledons. Pictures H to N (Fig. 3.8) show how random the pattern was in different shaped cotyledons, the most disruption occurring in fused cotyledons and those with more than one vascular strand entering the petiole.

Together these results show that the *hydras* have severely disrupted patterning of vascular tissues throughout the leaves, cotyledons and hypocotyl region, suggesting that the signalling events required for normal patterning are confused and disorientated, as the pattern seems to adapt to the severity of the individual mutant concerned.

3.9 Stomatal Patterning Defects are Highlighted by the *ACS1::GUS* Marker

Because vascular patterning within the abnormally shaped and developed cotyledons and leaves was abnormal, it was decided to briefly investigate another patterning mechanism within the leaves and cotyledons. Stomatal patterning is a well regulated and well researched area, and since the pattern is linked with the development of the

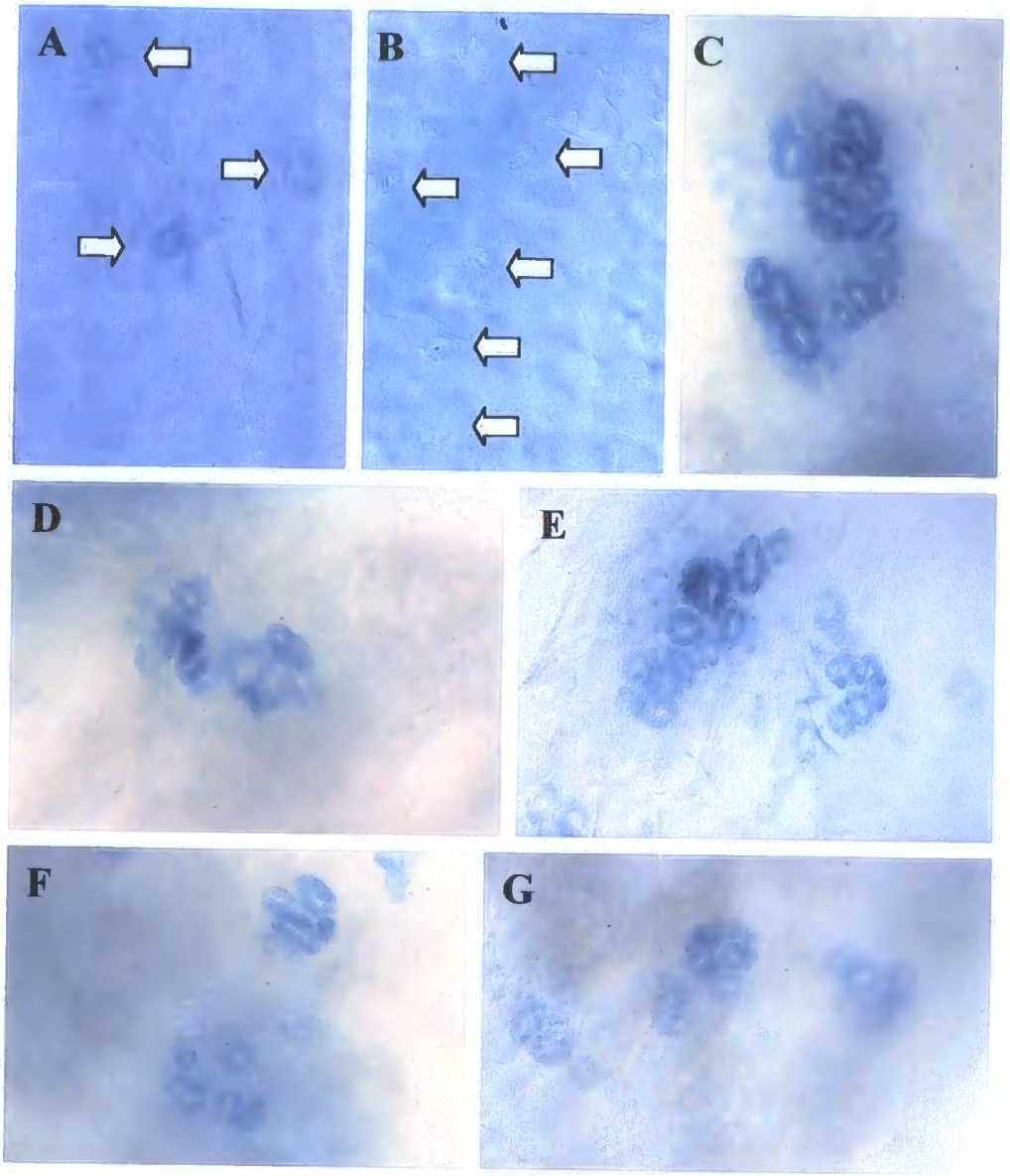
Figure 3.9

***ACS1::GUS* Stomatal Patterning**

A and B: Wild type expression of *ACS1::GUS* in the stomata of the leaf. White arrows indicate the spacing pattern of individual stomata in a wild type leaf.

C to G: *ACS1::GUS* expression in the stomata of a *hydra2* leaf.

Figure 3.9



tissues underneath the epidermis, such as the vascular tissues, it suggested that it would be an interesting thing to look at.

The *ACS1::GUS* marker line showed expression in the stomata of the cotyledons, leaves and hypocotyl, and so it was used to help visualise the stomata in the *hydra* mutants. Figure 3.9 shows the wild type patterning of stomata (Fig. 3.9, A, B and C), with individual stoma being evenly distributed across the leaf surface and interspersed with pavement cells. In *hydra2* however, they were found developing in clusters that were not evenly spaced or interspersed with pavement cells (Fig. 3.9, D-H).

3.10 The Hypocotyl Region: *LIPID TRANSFER PROTEIN1::GUS* Expression

An initial assessment of the mutants suggested that there was no clearly defined hypocotyl region, and that the majority of the upper part of the seedling consisted of an expanded shoot meristem. In order to address this concern a GUS marker line, *LIPID TRANSFER PROTEIN1::GUS* (*LTP::GUS*) (Vroeman *et al.*, 1996), that had previously been crossed into the *hydra1* background by Dr Jen Topping (University of Durham) but had not been assessed, was examined.

LTP1::GUS differentiates between the shoot and hypocotyl regions in the seedling because it stains from the shoulder of the cotyledons upwards, so that no staining is seen in the hypocotyl region (Figure 3.10, A). In *hydra1* there is clearly a region underneath the stained cotyledon shoulders that goes down to the root, indicating that there is a hypocotyl region in these mutants.

3.11 Radial Patterning Within the Hypocotyl is Disorganised and Defective

The presence of two or more vascular strands running through the hypocotyl shown by the *hydra1VT-1* double mutant suggested that the radial patterning of the hypocotyl could be disrupted as well. It was hypothesised that the radial pattern was either duplicated perfectly around the extra vascular poles or was lacking in organisation. In

Figure 3.10

LIPID TRANSFER PROTEIN1::GUS* Expression in *hydra1

A: *LTP1::GUS* expression in wild type, 7 DAG (mag. x 4).

B and C: *LTP1::GUS* expression in *hydra1*, 7 DAG (mag. x 4).

Figure 3.10

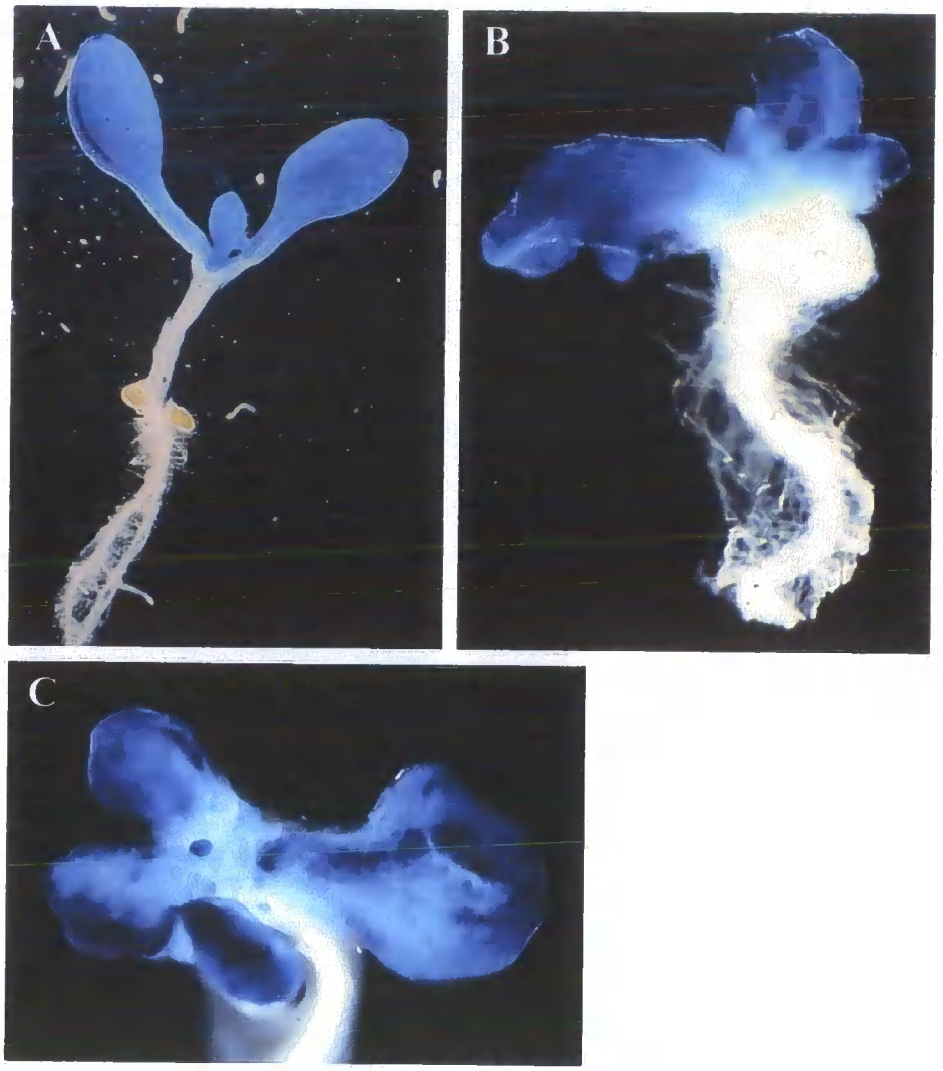


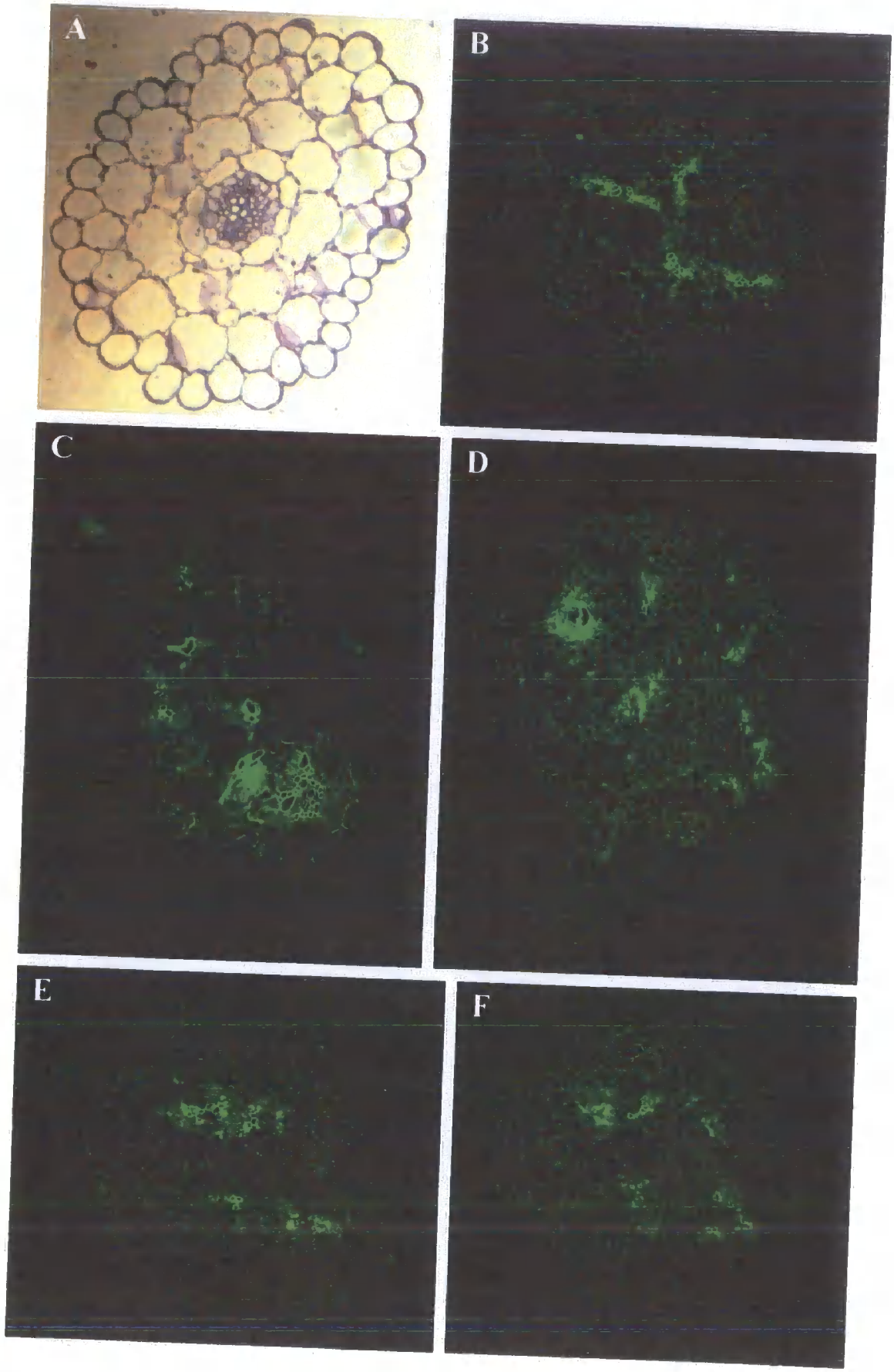
Figure 3.11

Transverse Sections of the Hypocotyl

A: Wild type hypocotyl in transverse section (mag. x 50).

B to F: Transverse sections of the *hydra2* hypocotyl stained with acridine orange (mag. x 40).

Figure 3.11



order to investigate this question of pattern, serial sections were cut through the hypocotyl of *hydra2* mutants.

Longitudinal sections through the hypocotyl showed that there was no correlation between the development of vascular strands and the pattern around it. Instead of aligned and connected vascular strands, the *hydra2* mutants showed numerous extra divisions within the vascular tissues and surrounding them (data not shown).

Transverse sections seen in Figure 3.11 show a variety of disrupted patterns, the severity of which was determined by the number of vascular poles running through the tissue. The wild type pattern through the hypocotyl consists of an epidermis, two cortical cell layers and an endodermal layer surround the pericycle and vascular tissues (Fig. 3.11, A). Multiple vascular strands in the *hydras* did not correlate with a duplicated pattern, indeed the whole pattern within the tissue was abnormal and unrecognisable. The sections of *hydra2* in Figure 3.11 were stained with acridine orange and captured on a confocal microscope. There were numerous extra cell layers and such disorganised cell divisions within this region, that there was no visibly obvious distinction between the endodermis, cortex or vascular cylinder. The numerous vascular strands were disorganised and instead of being surrounded by endodermal tissues, were surrounded by cells the appearance of cortical cells but with no specific characteristics.

These results suggest that the hypocotyl develops a disorganised and responsive pattern rather than a co-ordinated and regulated one.

3.12 Root Growth and Development

As reported earlier, the roots of both *hydra1* and *hydra2* were shorter than normal, and so it was important to further investigate the development of the roots. To achieve this a developmental timecourse was performed, with root growth assays and histological analysis being undertaken every three days after germination. Because of the plasticity between the development of the main and the adventitious roots, only the primary root was assessed in all experiments.

As Table 3.12 below shows, root growth in the *hydra* mutants was considerably reduced when compared with wild type (n = 10). There was no root growth seen for the first 3-4

days after germination. The wild type seedling root continued to increase in length at a steady rate throughout the assay. *hydra1* started slowly after day 3 such that by day 6 only a small amount of growth had occurred. After this point, however, *hydra1* accelerated and then continued at a steady rate until the death of the seedling (normally after about 30 days).

hydra2 root growth was not significantly faster than *hydra1* after 3 or 6 days, however by day 9 *hydra1* was longer than *hydra2*. Growth in *hydra2* continued, although slowly, and by day 12 had started to slow down so that root growth eventually ceased between days 15 and 18.

Table 3.12 Root Length (mm) ± s.e.

DAG	C24	WS	*hydra1*	*hydra2*
3	5.75 ± 0.61	6.321 ± 0.32	0.75 ± 0.09	0.39 ± 0.09
6	34.14 ± 1.31	39.68 ± 1.13	1.43 ± 0.15	2.07 ± 0.16
9	71.68 ± 3.9	92.93 ± 2.0	6.04 ± 0.24	3.18 ± 0.19
12	120.29 ± 5.23	136.0 ± 3.22	12.46 ± 0.72	3.68 ± 0.21
15	183.79 ± 6.06	189.0 ± 4.27	16.39 ± 0.76	4.14 ± 0.23
18			21.0 ± 0.89	4.25 ± 0.19
21			26.79 ± 1.05	4.29 ± 0.18

Figure 3.12.1 Comparison of *hydra* Root Growth with Wild Type

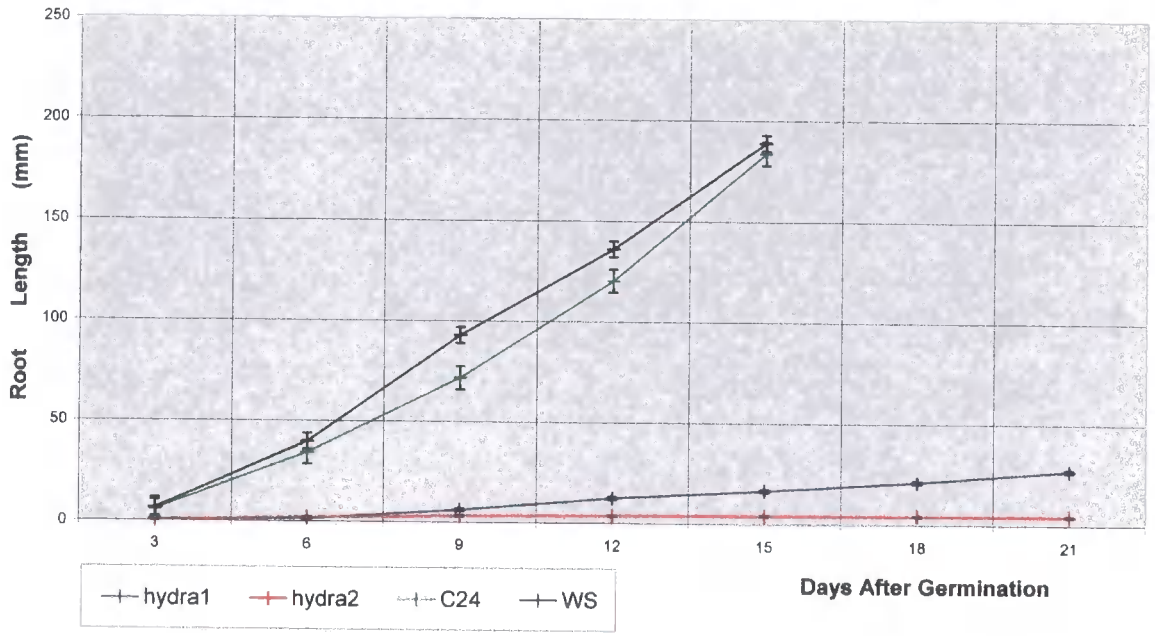
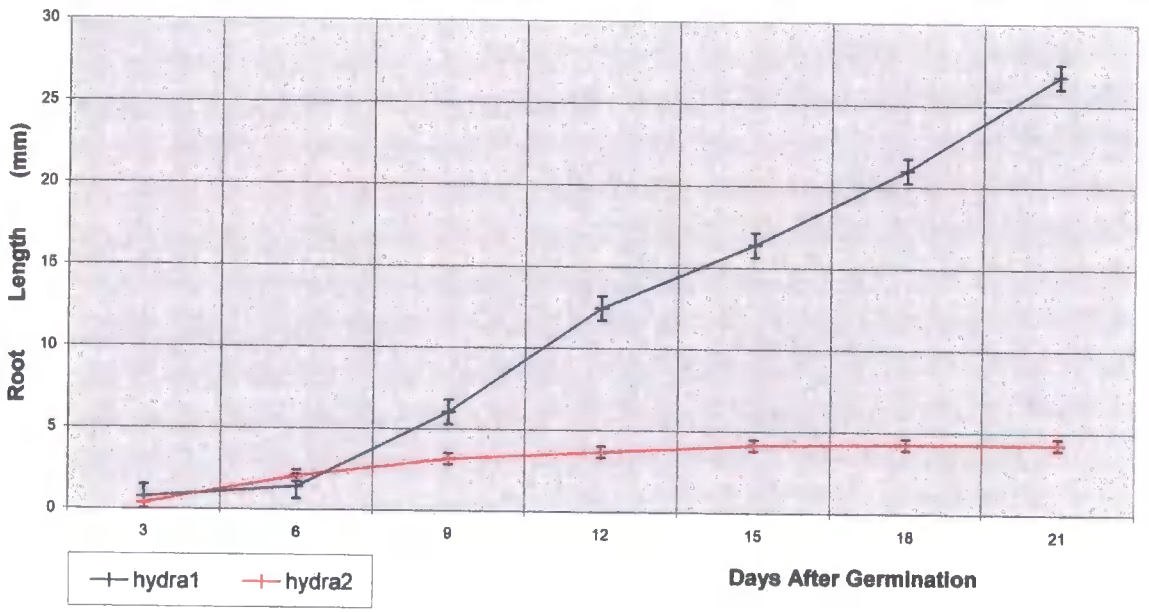


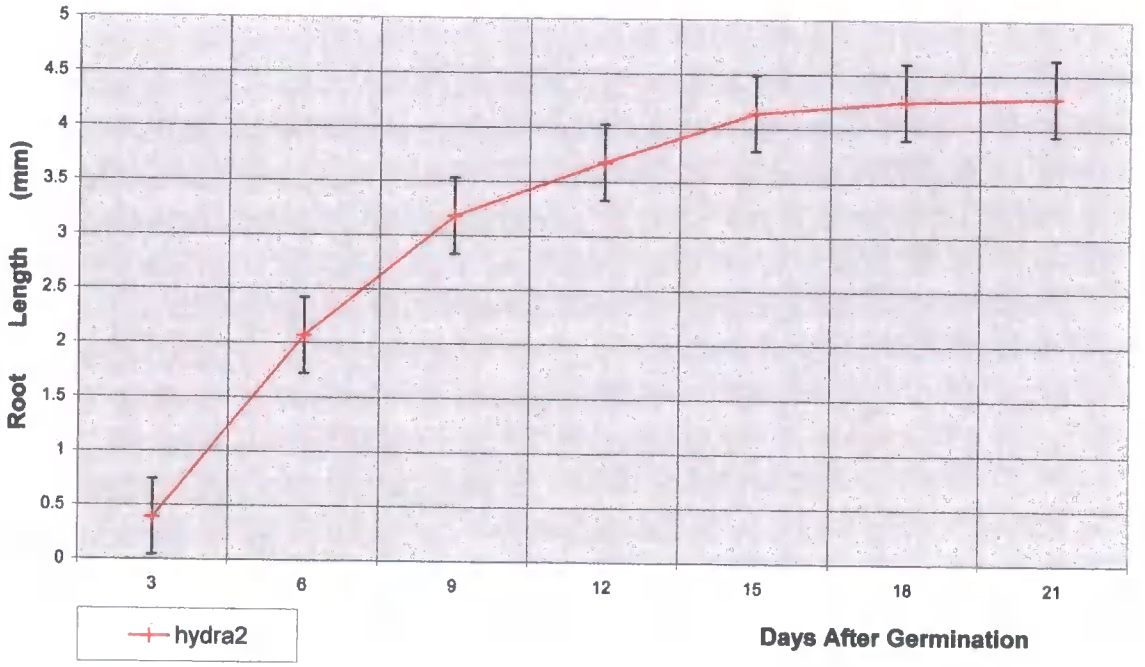
Figure 3.12.1 shows that both wild type varieties, C24 and WS, had a similar growth trend, whilst both *hydra* mutants showed reduced and distinctly different trends. When *hydra1* and *hydra2* root growth is compared more closely (Figure 3.12.2), the differences between the two mutants becomes clearer. Despite initially being shorter than *hydra2* at day 6, *hydra1* quickly overtakes *hydra2*, and then grows at a steady rate

Figure 3.12.2 Comparison of *hydra1* and *hydra2* Root Growth



until the end of the time course at day 21. *hydra2*, by comparison, grows at a steady, but reduced, rate until day 15 when it slows down and eventually halts at day 21. Figure 3.12.3 shows this declining root growth more closely.

Figure 3.12.3 *hydra2* Root Growth



The growth of the *hydra2* root shown in more detail above shows that its growth did not resemble the wild type accelerated growth trend, and instead showed some increase in root length followed by a reduction and eventual cessation of growth between days 15 and 18.

Although the *hydra2* primary root shows reduced and then halted growth, the lateral roots continue to grow so that the lateral roots growing off of the primary root overtakes it.

These results show that both *hydra* mutants exhibit defective root growth, with *hydra2* showing the more severe phenotype.

3.13 The Root Cap Columella is Lost During *hydra2* Root Growth

To follow up the observations made above, a closer look was taken at the main root meristem, and in particular the columella root cap. Lugol stains the starch granules present in the columella root cap that act as statoliths in the gravity response (Rosen *et al.*, 1999). In order to investigate further the reduced growth of the *hydra2* root, and to see if there was a concomitant loss of patterning at the same time as the growth ceased, lugol staining over a developmental time course was performed.

Figure 3.13 shows that the wild types (C24 and WS respectively) maintained a constant staining pattern throughout development (Fig. 3.13, A and B). As the cells are produced by the root meristem they become more distal to it and they start to differentiate into columella root cap cells. As they become columella cells they start to produce starch granules, each row of cells moving further towards the end of the root tip with every division of the initials in the meristem. The outermost layer is sloughed off as the root pushes through the soil.

hydra1, like the wild types, maintained a consistent staining pattern throughout development (Fig. 3.13, C, D and E), despite axis duplication having occurred in the main root (Fig. 3.13, J). *hydra2* had a normal staining pattern at 6 days old (Fig. 3.13, F and G), but then showed a complete loss of staining on day 14 (Fig. 3.13, H and I).

Figure 3.13

Lugol Staining of the Columella Root Cap

A: C24 wild type 14 days old.

B: WS wild type 14 days old.

C: *hydra1* 6 days old, staining normal.

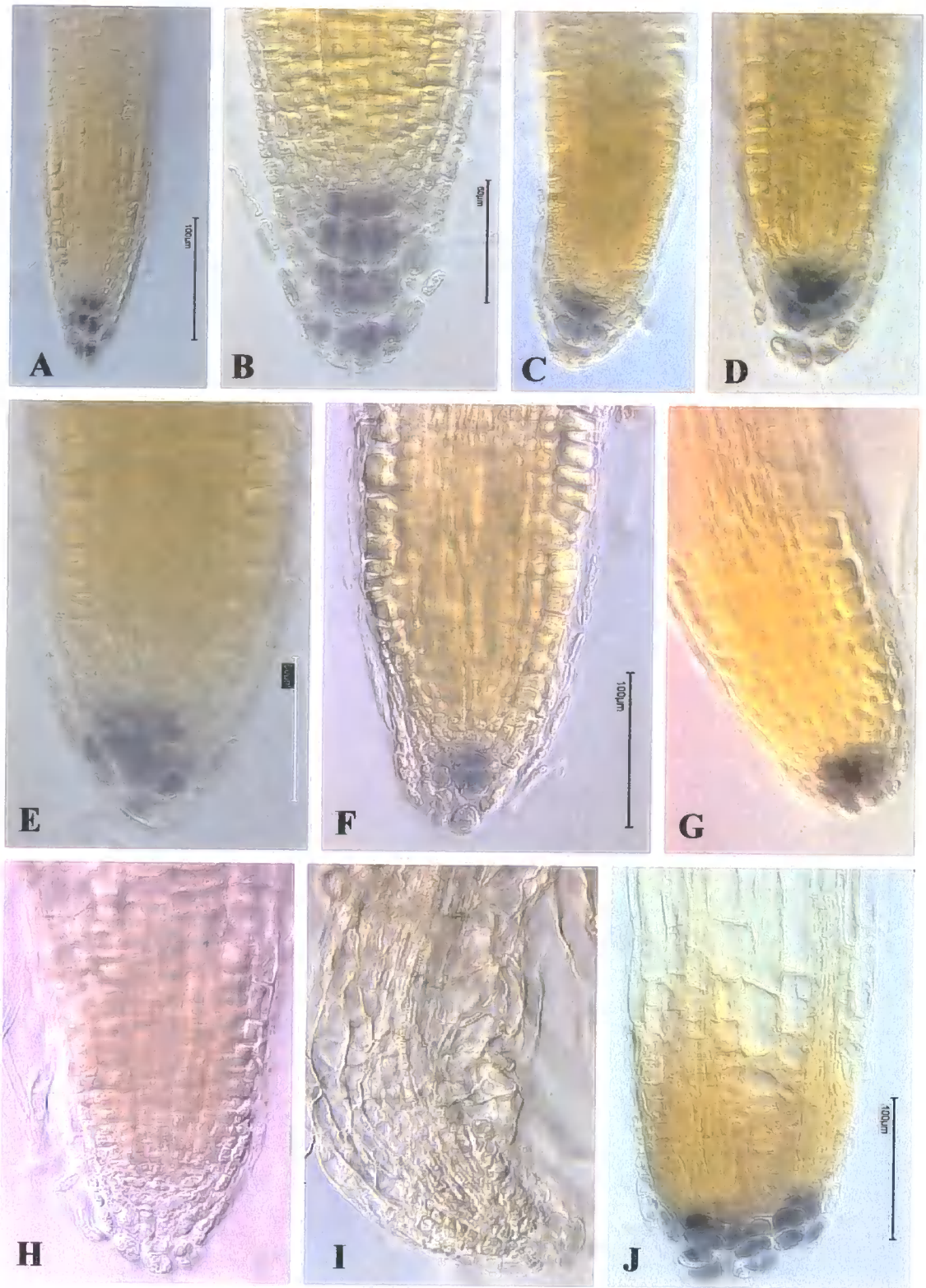
D and E: *hydra1* 14 days old, staining normal.

F and G: *hydra2* 6 days old, staining normal.

H and I: *hydra2* 14 days old, staining absent.

J: *hydra1* 14 days old, staining normal within a duplicated root.

Figure 3.13



These results clearly show that the *hydra2* root shows abnormal patterning resulting in the loss of the columella root cap. This may be due to a reduced amount of cell division within the meristem providing fewer initials for the columella, or because the signalling mechanism that defines the transition from initial to columella cell fate is disrupted.

These observations in *hydra2* are consistent with the root growth data in which the primary root starts to stop growing within this time frame, i.e. at days 12-18. *hydra1* however does not show this phenotype, and this is the main phenotypic difference between the two mutants. It is also interesting that the lateral roots in *hydra2* continued to stain with lugol having maintained their root caps, indicating that this problem is only restricted to the main root meristem.

3.14 Scanning Electron Microscope Analysis of the Root Tip

Scanning Electron Microscope (SEM) analysis of the root allowed a more detailed examination of at the root tip. Figure 3.14 shows wild type root tips (A and B) in comparison with *hydra2* (C to H) under the SEM. The root hairs initiate behind the root tip, in the differentiation zone, and in the wild type root tips there was a clear distance between the tip and the first initiating root hairs. In *hydra2* however, this distance was considerably reduced when compared with wild type, suggesting that there is either less cell division or less cell expansion within this region and so the differentiation of tissues starts closer to the root tip than in the wild type.

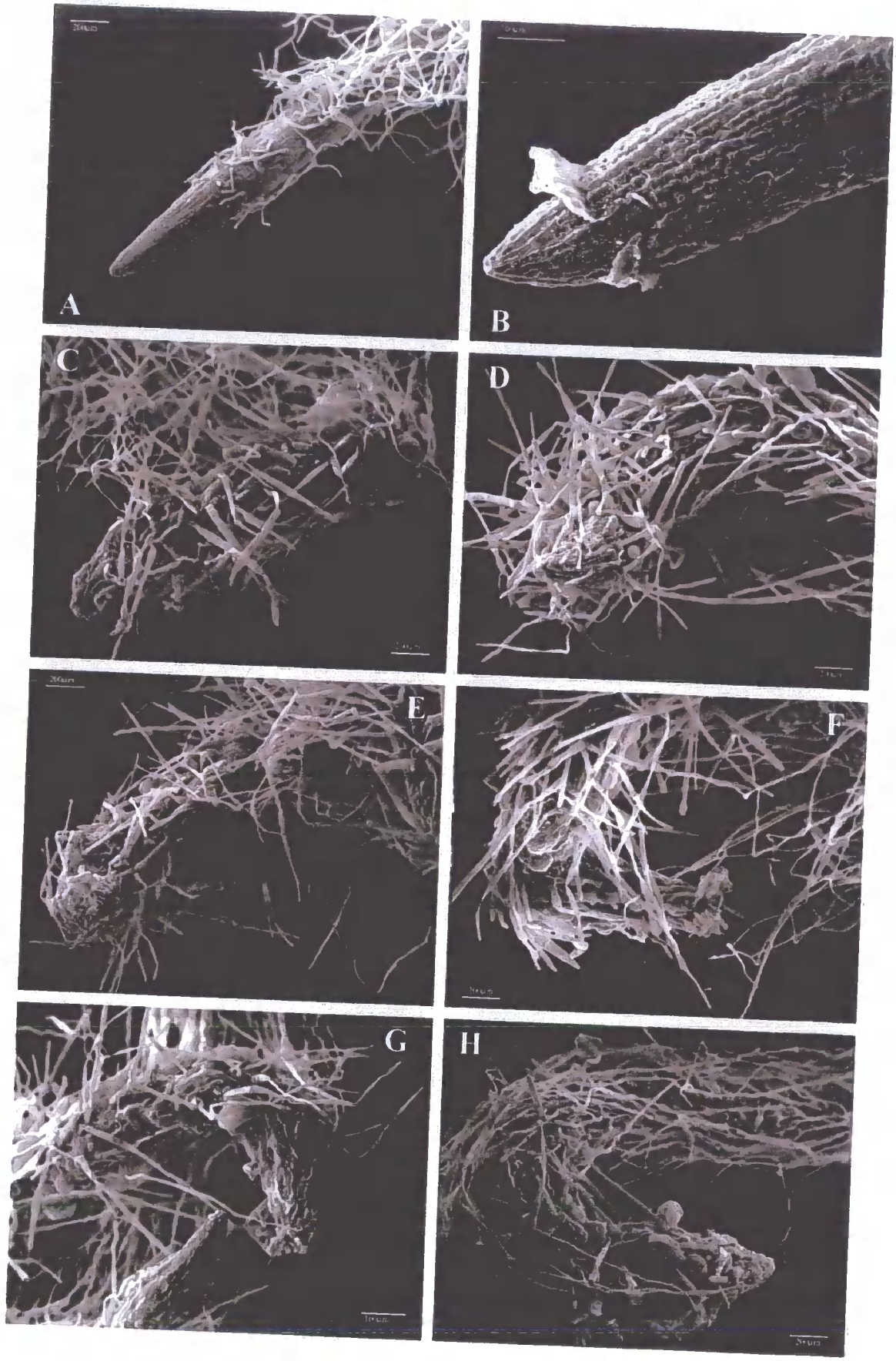
Figure 3.14

SEM Analysis of *hydra2* Main Roots

A and B: WS wild type 12 days old.

C to H: *hydra2* 12 days old.

Figure 3.14



3.15 *CYCAT1:CDB:GUS* Expression in the Primary Root of *hydra2* Diminishes with Age and Correlates with Reduced Root Growth

The root growth data from *hydra2*, as well as other observations such as the loss of the root cap and the initiation of root hairs close to the root tip, suggested that there was a problem with either cell division within the meristem or cell expansion. It was therefore important to be able to visualise, in an indirect manner, the activity of the root meristem, to determine whether there was any difference in cell division activity. The *Cyclin B CYCAT GUS* line presented the best way of achieving this (Hauser and Bauer, 2000). For more detail on this line section 3.4 above.

Expression of *Cyclin B CYCAT GUS* in wild type seedlings was seen in the region covering the meristem and entering into the expansion zone of the main root tip, with individual cells being clearly stained (Figure 3.15.1, A and B). This expression pattern was maintained throughout development. In the *hydra1* background the staining pattern was normal and was maintained throughout development (pictures not shown). In *hydra2* however, expression was normal until day 9 (Fig. 3.15.1, G and H), when there was a considerable up-regulation in the intensity and amount of staining. Staining was still seen in the main root on day 12 (Fig. 3.15.1, I), but by day 15 there was a considerable reduction in staining that was nearly absent (Fig. 3.15.1, J and K). By day 18 there was no detectable staining at all in the main root (Fig. 3.15.1, L, M and N). Consistent with the continued growth of the lateral roots, there was still a normal level of expression in the lateral root meristems.

These observations show that there is a reduction in cell division within the main root meristem during development, that fits into the same time frame of events as those mentioned previously; the reduction and eventual cessation of root growth and the loss of the root cap. Interestingly, there is an increased amount of cell division on day 9 followed by a reduction from day 12, ending in a complete lack of cell division by day 18.

The *Cyclin B CYCAT GUS* line also shows very clearly the cell divisions in developing lateral root primordia, with GUS visible from the first division of the pericycle. By using this line, observations made of lateral roots development in the *hydra* mutants.

Figure 3.15.1

***CYCAT1:CDB:GUS* Expression in the *hydra2* Root**

A and B: Wild type *CYCAT1:CDB:GUS* expression (A mag. x 20, B x 30).

C to N: *CYCAT1:CDB:GUS* expression in *hydra2* during development:

C and D (3 days old) (mag. x 20).

E and F (6 days old) (mag. x 20).

G and H (9 days old) (mag. x 30).

I (12 days old) (mag. x 20).

J and K (15 days old) (mag. x 20).

L, M and N (18 days old) (mag. x 20).

Figure 3.15.1

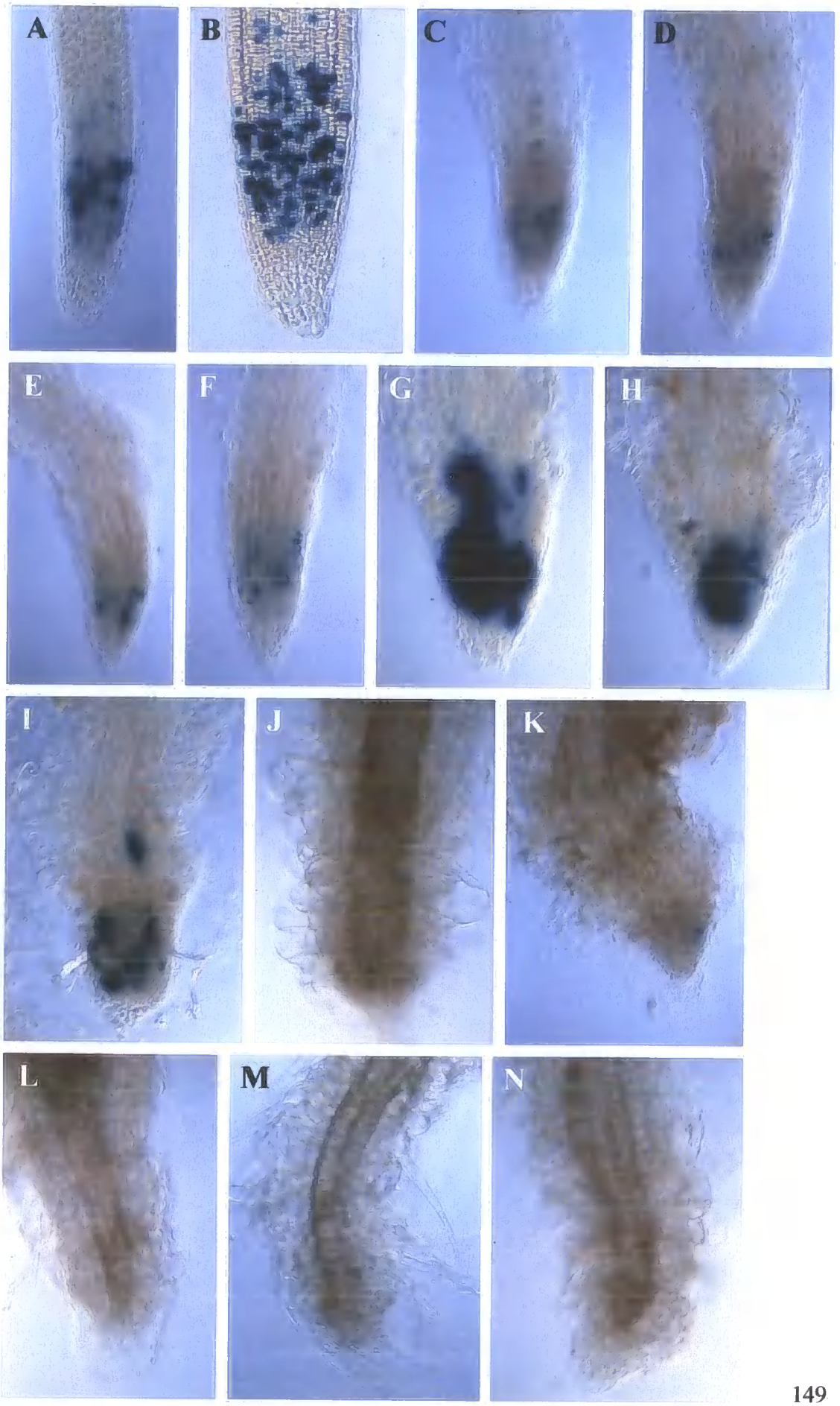


Figure 3.15.2

***CYCAT1:CDB:GUS* Expression in Lateral Roots**

A-G: Wild type *CYCAT1:CDB:GUS* expression at various stages of lateral root development (A mag. x 60, B x 80, C, D and E x 40, F and G x 20).

H-L: *CYCAT1:CDB:GUS* expression in *hydra2* at various stages of lateral root development (H mag. x 40, I, J, K and L x 60).

Figure 3.15.2

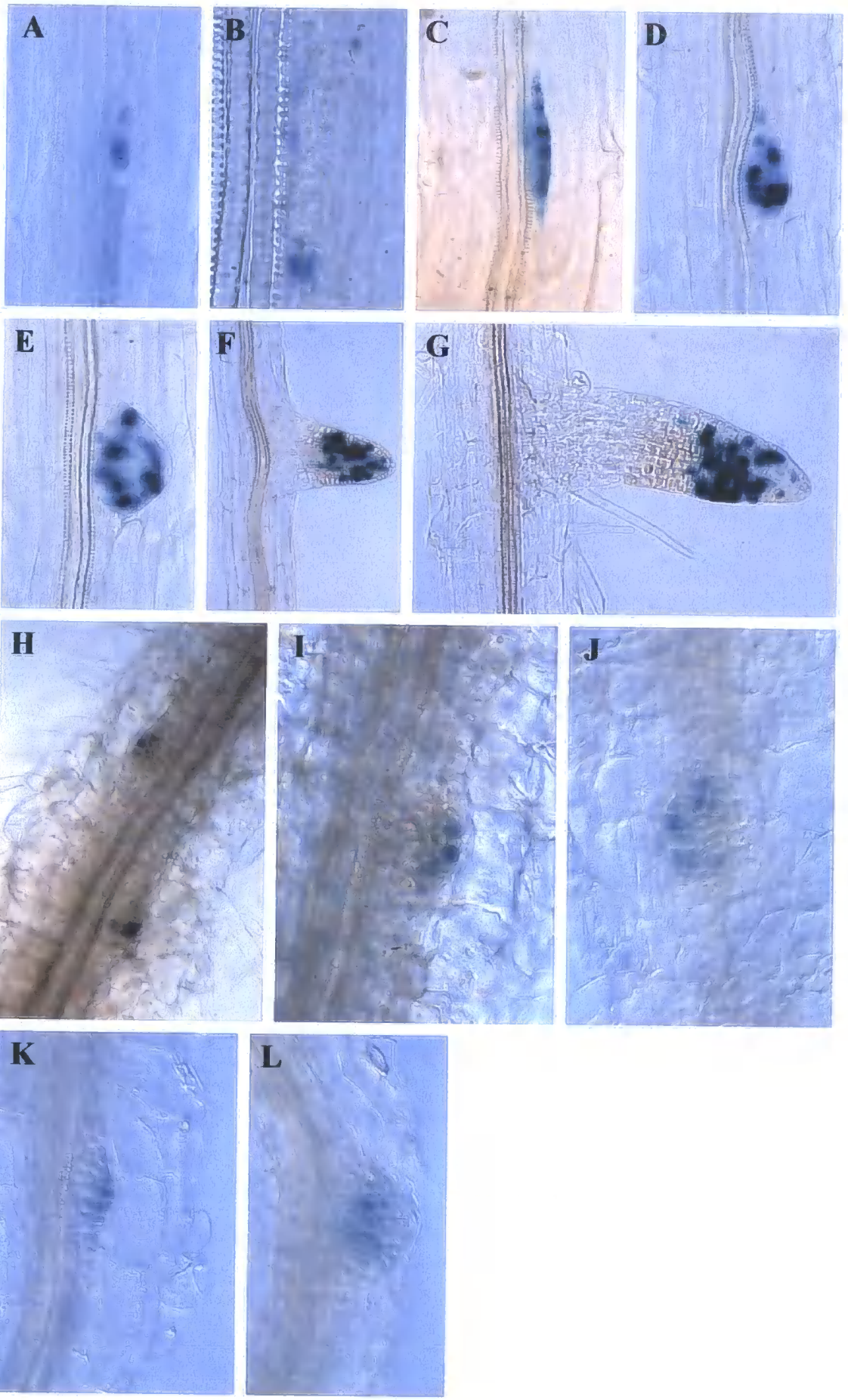


Figure 3.15.2 shows the normal pattern of staining in the wild type, which can be visualised from the initial founder cell divisions within the pericycle (Fig. 3.15.2, A and B), through the development of the primordium (Fig. 3.15.2, C, D and E), and into the emergence of the young lateral root, when it has similarity to the pattern of staining seen in the main root (Fig. 3.15.2, F and G). Consistent with published models of lateral root development, the lateral initiated opposite a protoxylem pole on one side of the root (Malamy and Benfey, 1997).

Lateral root initiation and development was found to be wild type in both *hydra1* and *hydra2*; Fig. 3.15.2, H and I clearly show that the lateral initiated opposite a protoxylem pole, although the two laterals developing in H have initiated close together. Fig. 3.15.2, J, K and L show that the expression pattern is normal in the developing and pre-emergence lateral.

3.16 Mutations in the *EXORDIUM* Gene Rescue Root Growth in *hydra2*

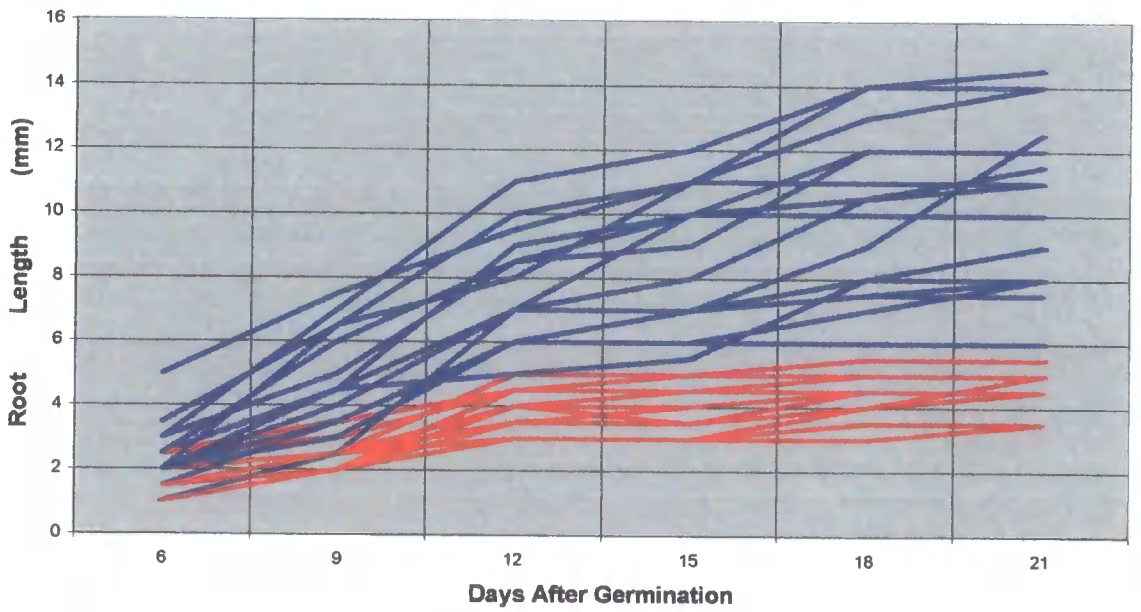
To investigate further the observation of abnormal cell division within the *hydra2* meristem, a second cell division marker was crossed in the *hydra2* background. The *EXORDIUM* (*EXO*) gene was tagged in the GUS AtEM201 promoter-trap line (Topping *et al.*, 1997). GUS expression in seen in the root meristem, and cloning of the *EXO* locus showed that the gene encodes a protein related to *PHOSPHATE INDUCED-1* (*PHI-1*). *PHI-1* protein was identified in a tobacco BY-2 cells (Sano *et al.*, 1999), being rapidly induced by the addition of phosphate to phosphate-starved cells in a state of cell cycle arrest. Sano *et al.*, (1999) suggest that *PHI-1* allows phosphate starved cells to re-enter the cell cycle. The *exo* line shows a considerably reduced level of normal transcript, however there is no phenotype associated with the line, which may be due to other homologous sequences in the genome giving functional redundancy (Farrar *et al.*, submitted).

AtEM201 plants homozygous for the *exo* mutation were crossed with *hydra2*. 30 F2 seedlings with the *hydra2* phenotype were screened for *EXO-GUS* expression and modified *hydra2* phenotypes. Each seedling was measured for root growth and then

individually GUS stained after 30 days growth, so that any phenotype associated with the presence of GUS (and therefore the *exordium* mutation), could be determined.

Figure 3.16.1 below shows all of the 30 seedlings plotted onto the same graph, all GUS-negative seedlings are represented in red, whilst all GUS-positive seedlings are white. Of the 30 seedlings, exactly two-thirds (20/30) were GUS positive, being either homozygous or heterozygous for the promoter trap *exo* mutation. All of the 20 GUS-positive *hydra2* seedlings were found to have a modified *hydra2* phenotype. These seedlings had longer roots than *hydra2* mutant seedlings and fell into two classes, based on root growth measurements (see Figure 3.16.2 and Figure 3.16.3 below). At ca. 21 days post germination, the GUS-negative *hydra2* seedlings had a mean primary root length of 4.3 ± s.e. 0.4mm. For one group of GUS-positive *hydra2* seedlings, the primary root length at ca. 21 days was 8.4 ± s.e. 0.6mm, while for a second group of GUS-positive seedlings the mean primary root length at ca. 21 days was 12.9 ± s.e. 0.2mm. This later group with the longest mean root length is predicted to represent *hydra2exo* double homozygotes, and the results are consistent with the *exo* mutation having a semi-dominant effect in suppressing the *hydra2* mutant phenotype.

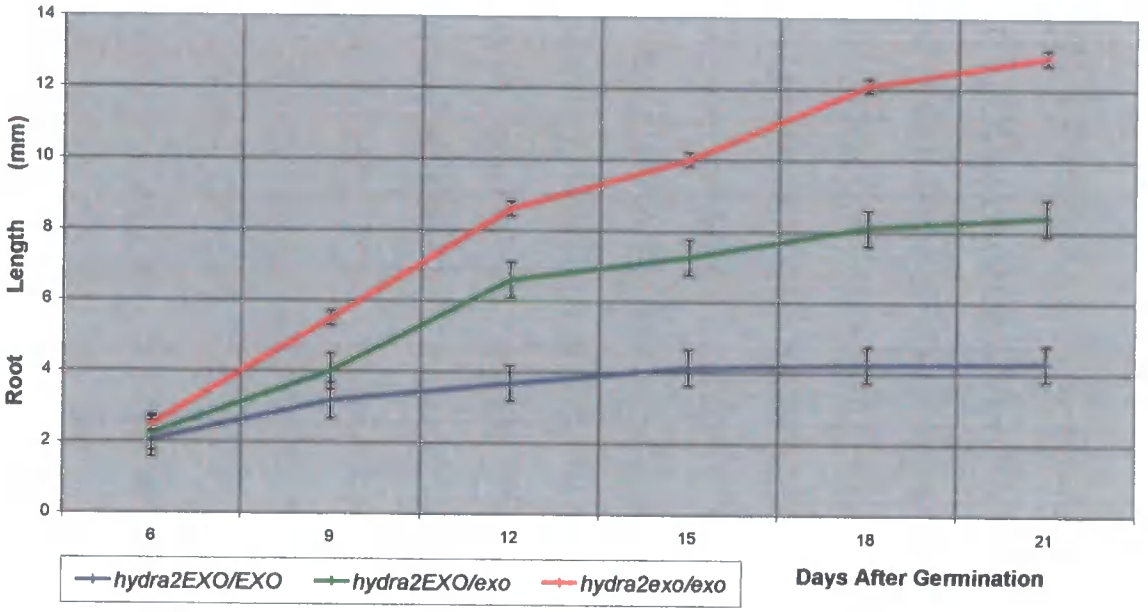
Figure 3.16.1 *hydra2exo* Root Growth and GUS Expression



The root of the predicted heterozygous *hydra2/hydra2EXO/exo* seedlings continued to grow steadily after day 21, although their overall growth was slowing down.

Microscopical analysis showed that the putative *hydra2/hydra2exo/exo* double mutants had a normally patterned meristem (Figure 3.16.4). Consistent with this observation, all but 2 of the GUS-positive seedlings still had staining in the main root at 21 days after germination (Fig. 3.16.4, L and M), showing that the *EXORDIUM* gene is associated with the observed phenotype. The double homozygous *hydra2/hydra2exo/exo* plants, meanwhile, continued to grow and did not show signs of slowing down.

Figure 3.16.2 *hydra2exo* Root Growth



These results indicate that *EXORDIUM* has a role in regulating cell division within the meristem, and that the *exo* mutation rescues the cell division activity within the *hydra2* meristem.

Figure 3.16.3

***hydra2EXORDIUM* Double Mutants**

A: (L-R) a *hydra2* mutant, a predicted *hydra2/hydra2EXO/exo* heterozygote, and a predicted *hydra2/hydra2exo/exo* homozygote, 18 DAG.

B: (L-R) predicted *hydra2/hydra2exo/exo* double mutant and a *hydra2* mutant, 18 DAG.

Figure 3.16.3

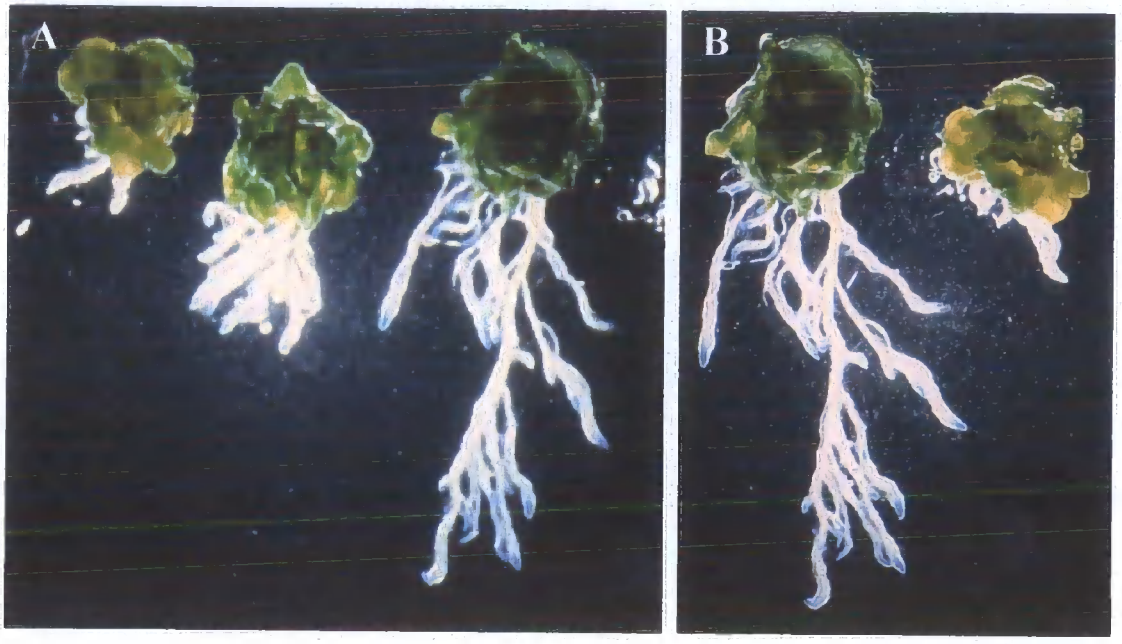


Figure 3.16.4

EXORDIUM* Expression in *hydra2

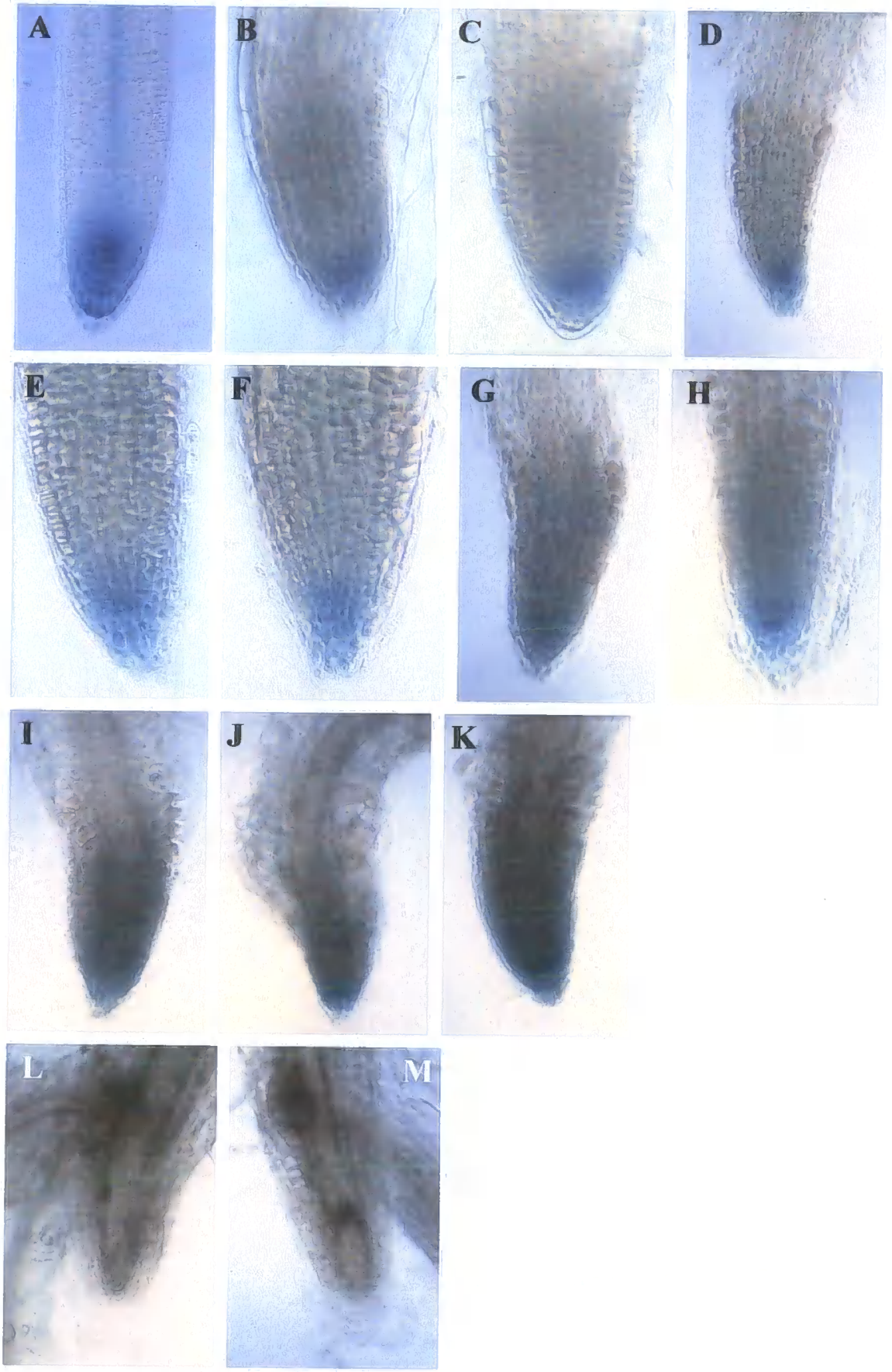
A: *EXO* expression in the promoter trap line EM201 (9 DAG), which represents the *exo* mutant (mag. x 20).

B to K: *EXO* expression in predicted *hydra2/hydra2exo/exo* double mutants at various stages of development (mag. x 20):

- B and C: 3 DAG
- D and E: 6 DAG
- F: 9 DAG
- G: 12 DAG
- H: 15 DAG
- I and J: 18 DAG
- K: 21 DAG

L and M: *EXO* expression in predicted *hydra2/hydra2EXO/exo* seedlings (21 DAG) (mag. x 20).

Figure 3.16.4



3.17 Root Hair Patterning is Defective and Development is Abnormal

Through the analysis of the root tip it was clear that root hairs were initiating closer to the tip than normal. In order to look closer at the development of root hairs in the mutants, light microscope and SEM analyses were undertaken.

In wild type roots the specification of root hairs is organised so that files of root epidermal cells form either hairs cells or non-hairs cells. The pattern is very uniform and predictable, hairs only form in epidermal cells (trichoblasts) that overly a junction wall of two underlying cortical cells. The predictable and stable pattern of wild type root hairs can be seen in Figure 3.17.1, where they start to develop behind the meristem and elongation zones of the root. In *hydra2* however, there was a reduced distance between the root tip and the first differentiating root hairs, consistent with observations mentioned above. Root hair fate was not restricted to specific cell files.

The appearance of the root epidermis was also different, epidermal cells were often swollen and had root hairs that had diverged from normal tip growth (Fig. 3.17.1, H, I, J, K and L). Root hairs initiated in a polar fashion, at the apical (root tip) end of the trichoblast, and this polarity was not disturbed.

Closer inspection of the *hydra* root epidermis at the SEM level showed the extent of the abnormal epidermal surface. Image A in Figure 3.17.2 shows the normal wild type pattern, whilst B and F show multiple root hairs developing from the same cell in *hydra1*, and C and D a more severe swollen epidermis in *hydra2*. Accompanying the swollen epidermis in *hydra2* was extreme ectopic root hair patterning, and development of more than one root hair from the same cell. Branched root hairs were also seen in both *hydra1* and *hydra2*.

These observations show that the *hydra* mutants have an extreme root hair phenotype, that is disrupted on many levels; cell specification and cell identity, localisation of out growth, and control of tip growth.

Figure 3.17.1

Light Microscope Analysis of Root Hair Development in *hydra2*

A, B and C: Wild type root hair patterning (A mag. x 8, B x 10, C x 12).

D – G: Root hair patterning in *hydra2* (mag. x 15).

H – L: Epidermal 'bubbling' in *hydra2* (mag. x 35).

Figure 3.17.1

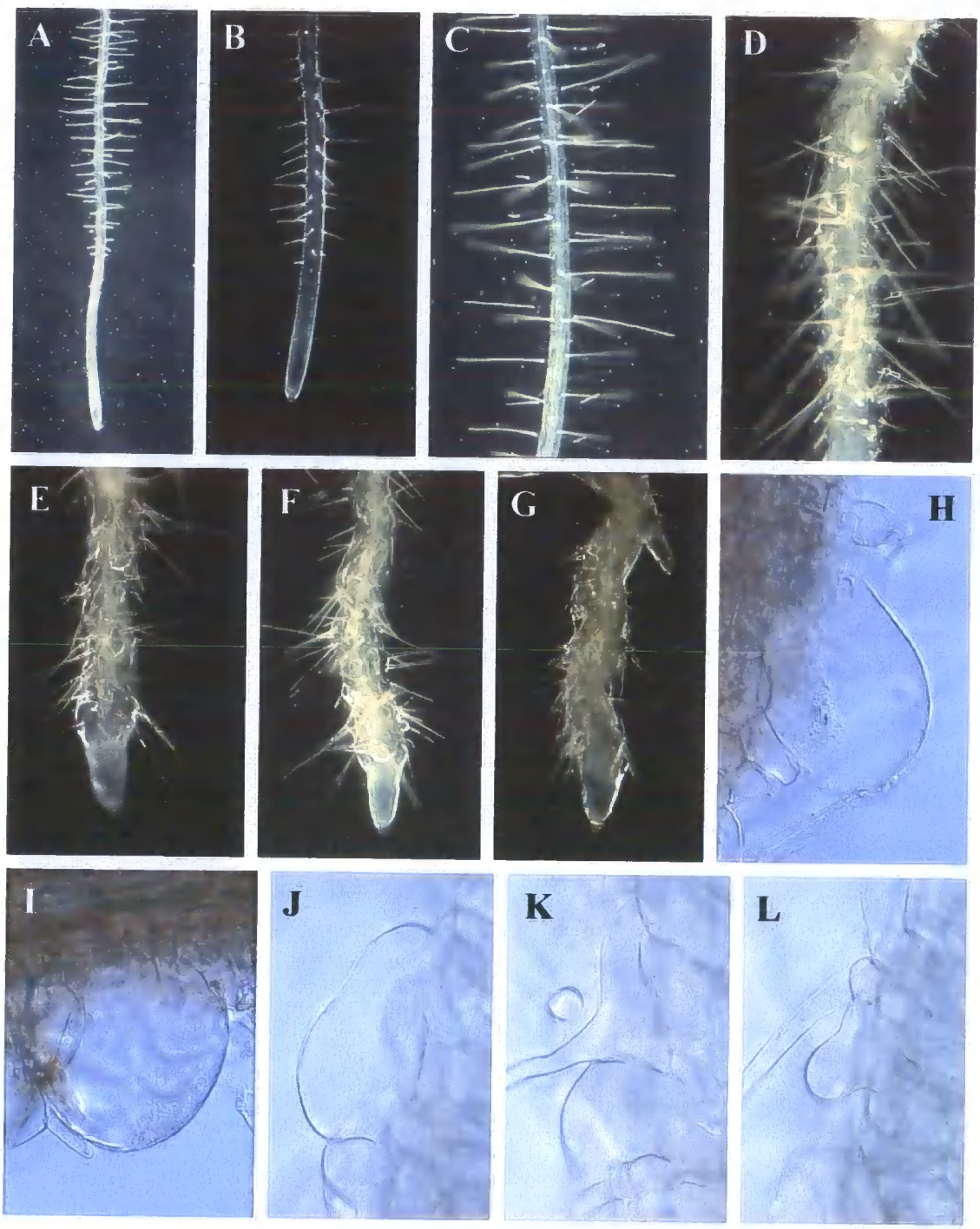


Figure 3.17.2

SEM Analysis of Root Hair Development

A: Wild type root hair patterning.

B: *hydra1* showing multiple root hair initiation sites.

C – F: *hydra2* showing multiple root hair initiation sites, swollen epidermal cells and ectopic root hairs.

G: *hydra1* root hair showing divergent tip growth.

Figure 3.17.2

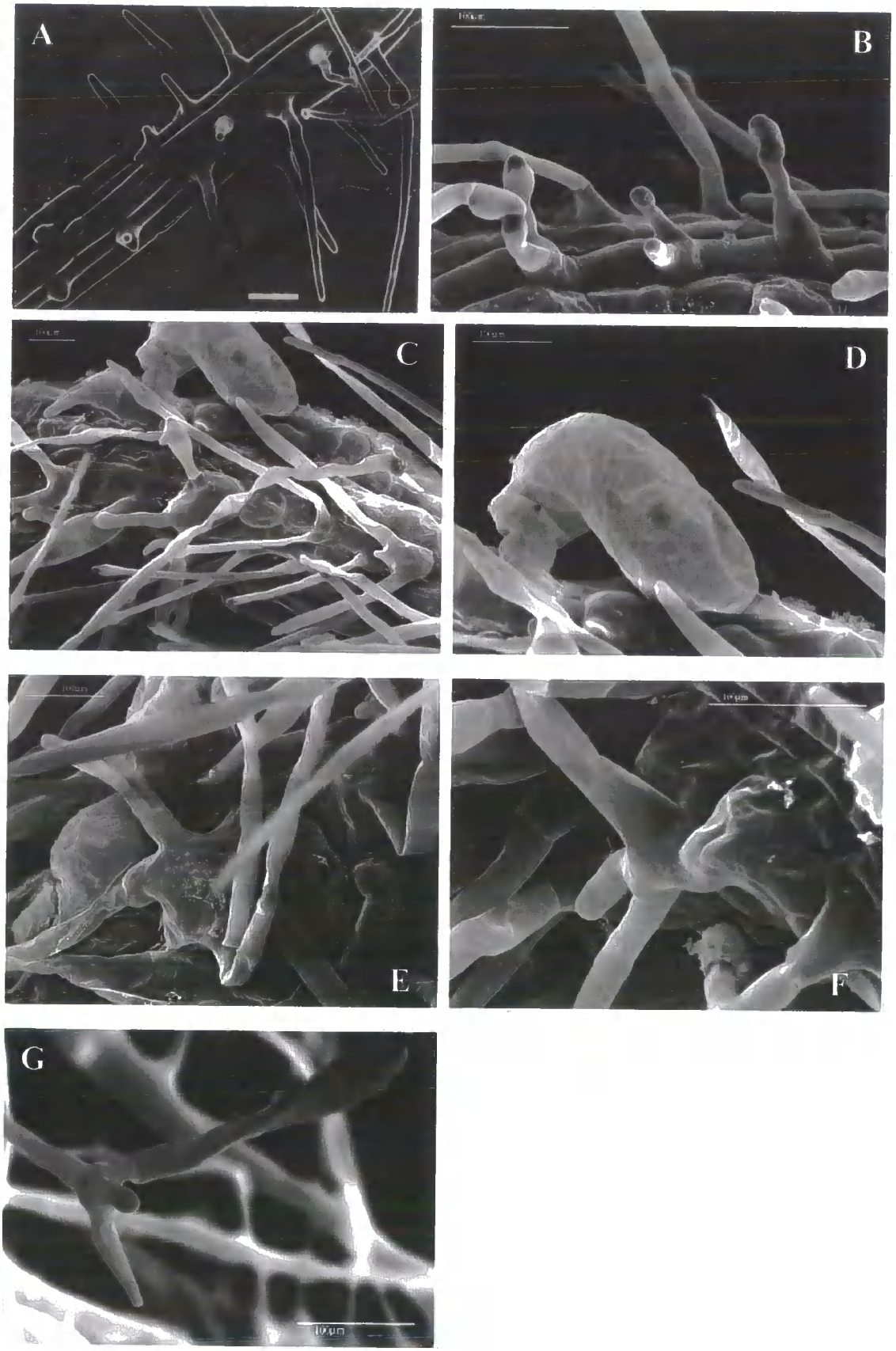


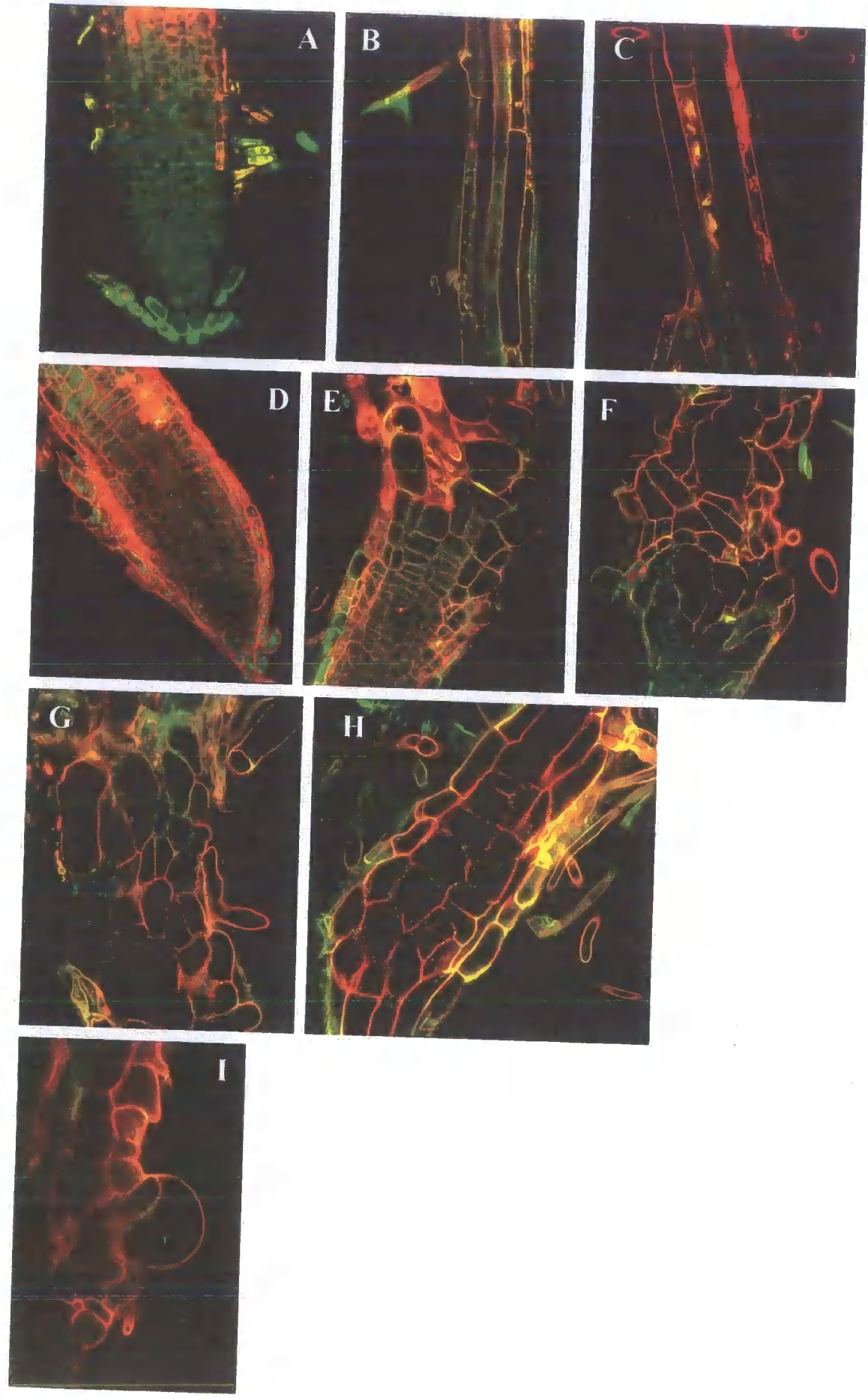
Figure 3.18

GLABRA2::GFP* Expression in *hydra2

A, B and C: Wild type expression of *GL2::GFP* (A mag. x 20, B and C x 30).

D to I: *GL2::GFP* expression in *hydra2* (D mag. x 20, E, F, G and H, I x 35).

Figure 3.18



3.18 *GLABRA2::GFP* Expression is Abnormal in *hydra2*

In order to investigate the molecular basis for the ectopic growth of root hairs, a *GLABRA2::GFP* line (courtesy of John Schiefelbein) was crossed into the *hydra2* background. *GLABRA2* is a homeobox gene that is required for specification of the hairless cell fate in atrichoblasts in the root epidermis, acting as a negative regulator of root hair development (Masucci *et al.*, 1996).

In a wild type background, *GL2::GFP* is expressed throughout the root tip, but is then restricted to the non-hair cell files (atrichoblasts) in the root hair differentiation zone of the root (refer to Figure 3.18). In *hydra2* expression was normal at the root tip, however there was no restriction of the expression once in the differentiation zone. GFP was also seen in the swollen epidermal cells.

These observations are contradictory to the phenotype seen, as this represents extreme ectopic expression of a negative regulator of root hair development. If the expression pattern was reflected in the phenotype then the roots should be hairless. Hormones such as auxin and ethylene are known to act downstream of *GL2* (Masucci and Schiefelbein, 1996), and these observations suggest that hormones might be overriding the expression of *GL2* and therefore causing the observed root hair phenotype.

3.19 Conclusions

Evidence has been presented in this chapter of a highly pleiotropic phenotype, some characteristics of which have been reported before for the *hydra1* phenotype (Topping *et al.*, 1997), while some have not been previously recognised or explored. Indeed, the isolation of *hydra2* has benefited the study of *hydra1* because it allows direct comparisons to be made, and as a consequence has shown a number of differences between the two mutants.

The *hydra1* and *hydra2* mutant phenotypes are almost identical. Like *hydra1*, *hydra2* is dwarfed with multiple cotyledons and is seedling-lethal. Both mutants are unable to regulate cell size and shape, and fail to undergo correct morphogenesis during embryonic and post-embryonic development. While apical-basal pattern elements are present (shoot, hypocotyl, root), multiple leaf-like cotyledons are produced, radial

pattern is defective, with supernumerary cell layers and aberrant vascular patterning. Root hair patterning is also abnormal and contradicts the expression pattern of the *GL2* homeobox gene.

A distinction between the *hydra1* and *hydra2* mutants is the more severe root-defective phenotype of *hydra2*. While the *hydra1* root is short when compared to wild type, they continue to grow very slowly until the seedling dies, at ca. 30 - 40 days post-germination (Topping *et al.*, 1997). In contrast, the *hydra2* root invariably stops growth completely at ca. 15 – 18 days post-germination. Microscopical examination shows a loss of correct cell patterning of the *hydra2* meristem and root cap columella.

The similarities in the mutant phenotypes suggests that the *HYDRA1* and *HYDRA2* genes may have similar functions. Many of the characteristics are suggestive of a number of different hormone signalling problems, such as defects in auxin and ethylene affecting root hair growth, and in auxin transport essential for the position and outgrowth of cotyledons and the elucidation of the subsequent phyllotaxial pattern. It is therefore possible that by understanding better the defects in these hormone signalling pathways in the mutants, we will gain more insight into the roles of the *HYDRA1* and *HYDRA2* genes.

The following results chapters will therefore look at the nature of the *HYDRA2* gene as well as further investigate a number of these hormone signalling pathways. Because of the complex and extensive phenotype, the root will be used as the primary focus of the investigations.

Chapter 4

Results:

The *HYDRA2* Gene

Introduction

A genomic fragment of 2.6 kb flanking the T-DNA in *hydra1-2* (Errampalli *et al.*, 1991) was cloned by plasmid rescue (Behringer and Medford, 1992) by Dr. Jen Topping (University of Durham; Souter *et al.*, 2002), and found to be 100% homologous to the 5' region of an EST which was identified as a cDNA encoded by the *Arabidopsis* Δ8-Δ7 sterol isomerase gene (Grebenbok *et al.*, 1998).

Combined with the results from the first chapter, in which it is clear that both *hydras* have near identical phenotypes, the cloning of *HYDRA1* suggested that *HYDRA2* may also function in sterol biosynthesis. The remainder of this chapter will look at the experiments carried out to investigate the nature and function of the *HYDRA2* gene.

4.1 Elucidation of the *HYDRA2* Gene: *hydra2 fackel* Allelism Test

Some time after the cloning of *HYDRA1*, two independent papers were published presenting the results of the cloning of another sterol biosynthesis gene. The *Arabidopsis FACKEL* gene encodes the sterol reductase enzyme that lies directly upstream from the *HYDRA1* sterol isomerase in the biosynthetic pathway (see Figure 4.1 for a layout of the pathway) (Jang *et al.*, 2000; Schrick *et al.*, 2000).

Because of the phenotypic similarities between the *hydras* and the *fackel* mutant, Jen Topping (University of Durham) had previously checked *hydra1* for allelism with *fackel* when *hydra1* was first isolated. However *hydra2* had not been checked for allelism, and so it was therefore important to ascertain if *fackel* and *hydra2* were mutations in the same gene.

The allelism test involved crossing two heterozygous parent plants (*FACKEL/fackel* and *HYDRA2/hydra2*), and then checking the F1 generation for mutants, their presence indicating that the mutations lie within the same gene. If no mutants were found in the first generation then this would indicate that the mutations lie within different genes, and the F1 would be heterozygous for both loci. Three separate individual parent couples, designated A, B and C (see Table 4.1 below), were taken and crossed using each as male and female parents in a reciprocal cross. The F1 seed was collected, dried out, and then surface sterilised and plated out onto agar under sterile conditions. After

vernalisation seeds were allowed to germinate and the number of mutant and wild type seedlings were scored for each plate, ca. 7 days after germination. The results of this test are shown in Table 4.1 below.

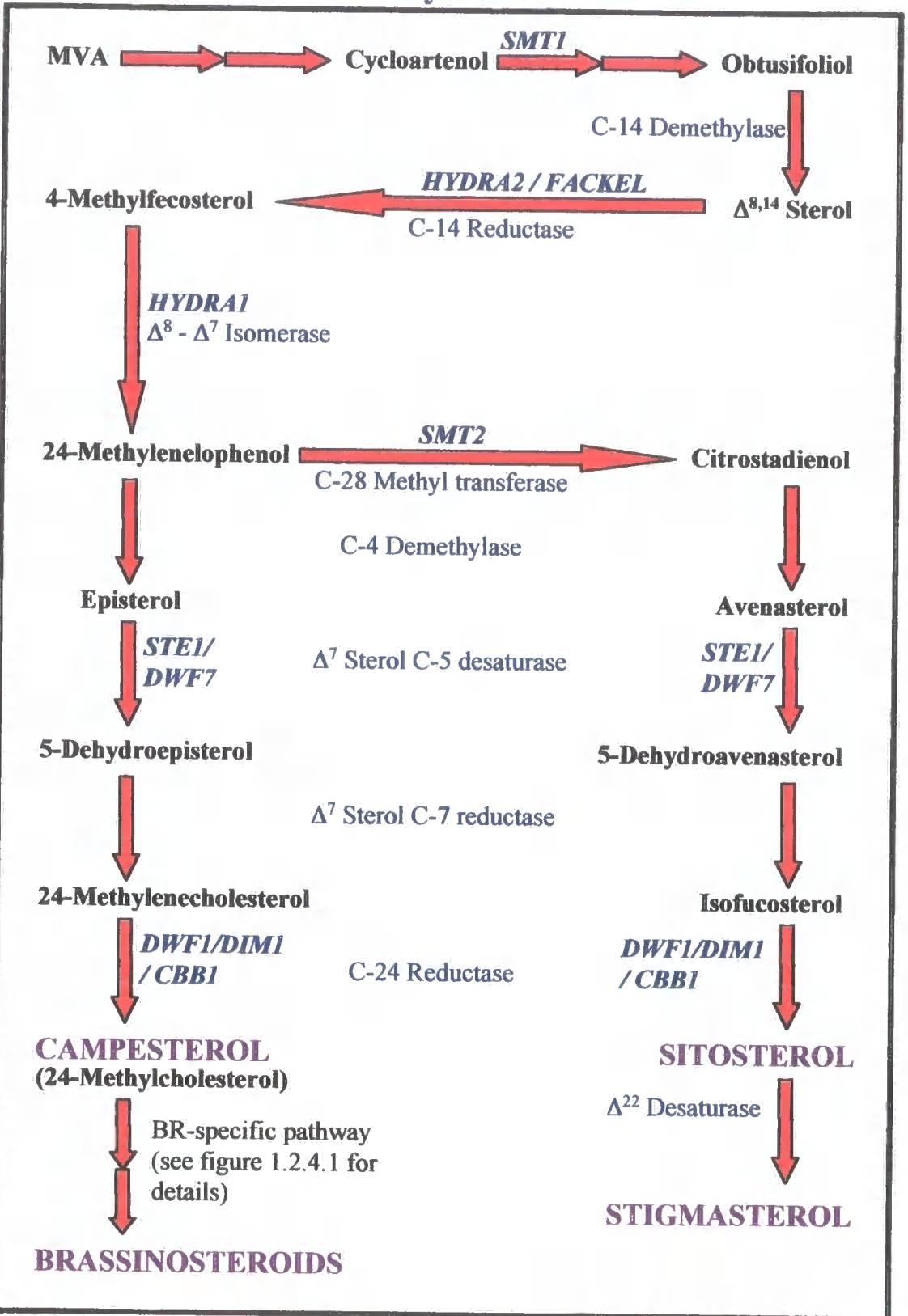
Table 4.1 Allelism Test			
Parents Couples		Seedlings	
Female	Male	Wild Type	Mutants
hydra2 A	*fackel* A	25	7
fackel A	*hydra2* A	41	11
hydra2 B	*fackel* B	29	6
fackel B	*hydra2* B	32	5
hydra2 C	*fackel* C	22	2
fackel C	*hydra2* C	11	1
TOTAL		**160**	**32**
RATIO		**3.3**	**0.7**

The presence of mutants in the F1 generation indicated that *hydra2* and *fackel* are allelic, and therefore represent mutations in the same gene; *HYDRA2/FACKEL* encodes a C14 sterol reductase. The wild type and mutant seedlings segregated approximately as a 3 to 1 ratio, which is consistent with the recessive nature of this mutation.

4.2 Sterol Analysis Confirms a Role For These Genes in Sterol Biosynthesis

It was predicted that the *hydra* mutants would have abnormal sterol levels present because of the role of the two genes within the biosynthetic pathway. Therefore, the concentrations of the three major membrane sterols, campesterol, sitosterol and stigmasterol, were compared. Triplicate pooled samples of extracts of *hydra* mutants and wild-type seedlings (10 DAG) were subjected to gas chromatography analysis by the commercial Lipid Analytical Service at the Scottish Crops Research Institute, Dundee. Mean sterol concentrations in wild-type (μg/g FW tissue) and *hydra1* and *hydra2/fackel* mutants (as a % of wild-type concentration) are shown in Table 4.2 below.

Figure 4.1 Plant Sterol Biosynthesis



Key: Enzymes catalysing these reactions and ***Arabidopsis* genes corresponding to these enzymes.** Multiple arrows represent more than one biosynthetic step not included here. Taken from Schrick *et al.*, (2000) and Jang *et al.*, (2000).

Table 4.2 Sterol Analysis			
Sterol	Wild Type	% of Wild Type Levels	
	µg sterol /g FW	*hydra1*	*hydra2*
Campesterol	4.2	12%	0%
Sitosterol	19.51	2%	4%
Stigmasterol	1.62	182%	322%

Both *hydra1* and *hydra2* had much reduced levels of sitosterol and campesterol, with elevated levels of stigmasterol, consistent with observations made of the *fackel* mutants (Jang *et al.*, 2000; Schrick *et al.*, 2000). These results confirm the role of these genes in sterol biosynthesis, and demonstrate that mutations in the sterol isomerase (in *hydra1*) and the C14 reductase (in *hydra2/fackel*) result in similar gross changes in sterol profiles in *Arabidopsis*.

4.3 RT-PCR Shows No Expression of the *FACKEL* Gene in *hydra2* Mutants

A initial T-DNA segregation analysis showed that there was no clear segregation of kanamycin resistance with the *hydra2* phenotype (A. Harrison and K. Lindsey, unpublished data), indicating that there was more than one T-DNA present in the *hydra2* line. Therefore, to investigate the expression of the *FACKEL* gene in the *hydra2* line, RT-PCR analysis was carried out. RT-PCR involves the purification of RNA for cDNA synthesis, as a substrate for PCR amplification using specific primers. Specific transcripts can therefore be identified and their expression analysed.

RNA was extracted from *hydra2* mutants and from WS seedlings and cDNA was synthesised. Specific amplification of the 1kb *FACKEL* transcript was achieved using primer combinations suggested by Kathryn Schrick (University of Tuebingen). As Figure 4.3 shows, the wild type WS seedlings express the *FACKEL* transcript, indicating that there are no problems with the specificity of the primers. In the *hydra2* seedlings however, no detectable *FACKEL* transcript could be seen. This observation shows that the *hydra2* mutation had disrupted the production of a normal *FACKEL* RNA transcript, and is consistent with a possible T-DNA insertion within the *HYDRA2* gene.

Figure 4.3

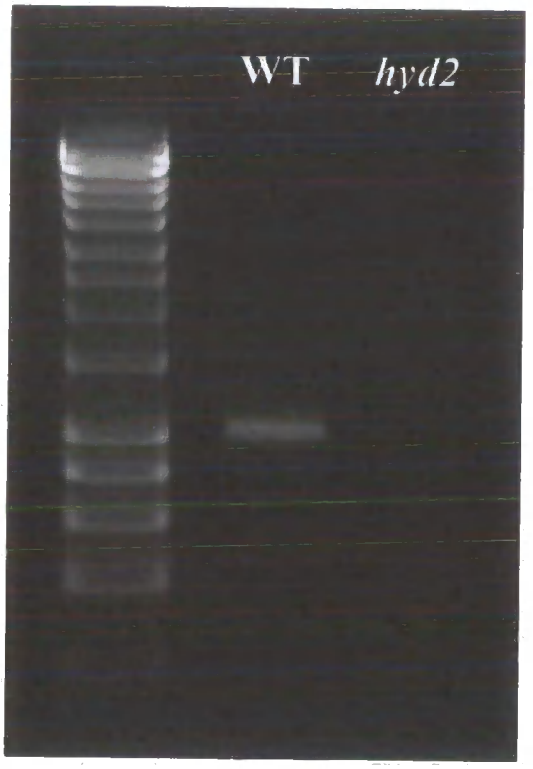
RT-PCR of the *FACKEL* Transcript in *hydra2*

The gel image shows the results of an RT-PCR experiment designed to amplify the *FACKEL* transcript.

(L-R) the ethidium bromide stained gel shows, 5µl of Hyperladder I (bioline.com), 10µl of WS wild type reaction products, 10µl *hydra2* reaction products.

The gel shown is the result of an initial amplification of the transcript followed by one round of nested primer amplification. The wild type track shows the expected 1kb product, the *hydra2* track does not show any transcript present, which was also the case after the initial amplification.

Figure 4.3



4.4 Brassinosteroid Treatment Does Not Rescue the *hydra* Mutant Phenotype

Sterols are known to contribute to two biosynthetic pathways in plants: the brassinosteroids (BRs) and the bulk sterols (Clouse and Sasse 1998; Hartmann, 1998). A number of mutants in plant sterol biosynthetic genes have been identified, most of which until recently were found to encode enzymes required for the synthesis of BRs. Such mutants are typically dwarfed and can be phenotypically rescued by exogenous BR application (e.g. Szekeres *et al.*, 1996; Choe *et al.*, 1999a,b).

Original analysis of *hydra1* showed that it was unable to be rescued by epibrassinolide application (Topping *et al.*, 1997). So, in order to see if the *hydra2* mutants were rescued by BR application, seedlings were grown in the presence of 1μM and 10μM epibrassinolide. BRs have been implicated in the induction of the *cycD3* gene which regulates the G1/S phase transition in the cell cycle. In order to see if BRs could rescue the cell division activity in the main root of *hydra2*, *hydra2CYCAT:CDB:GUS* double mutants were grown in the presence of epibrassinolide from 6 days post germination to 18 days post germination, when normal GUS expression and therefore cell division ceases.

The response of *hydra1* to BR application was previously described (Topping *et al.*, 1997), and *fackel* mutants have also been shown to be unresponsive to BR application (Jang *et al.*, 2000; Schrick *et al.*, 2000). Consistent with these results *hydra2* showed no recovery of any of the phenotype, or of *CYCAT:CDB:GUS* expression (pictures not shown), and so are similarly not rescued by BR application.

4.5 Conclusions

Evidence has been presented in this chapter to show that the *hydra2* mutant is defective in sterol biosynthesis, and is allelic to the *fackel* mutant. The *HYDRA2/FACKEL* gene encodes a C14 sterol reductase, a sterol biosynthetic enzyme that lies immediately upstream of the sterol isomerase enzyme encoded by *HYDRA1*.

Consistent with a proposed T-DNA insertion into the *hydra2* gene, no detectable transcript could be found using RT-PCR, and suggests that the insertion event may have caused a null mutation.

Sterols form the basis of two key components of plant growth and development. Firstly they are the precursors of the brassinosteroid hormones, and secondly they provide bulk membrane sterols. Brassinosteroids cannot rescue the phenotype of *hydra1* or *hydra2*, and Cyclin B expression is not rescued in the *hydra2* root meristem.

Bulk membrane sterols play a number of key roles within the membrane, they help to regulate fluidity and permeability, as well as interact with membrane-bound proteins and modulate their activity. Considering the fact that these two mutations lie early in the pathway before the brassinosteroid branch and before the production of the bulk sterols, it is possible that the cause of the pleiotropic phenotype could be due to membrane abnormalities rather than brassinosteroid signalling.

The next two chapters will present evidence of other signalling pathways that may be disrupted within the *hydra* mutants, in particular looking at cellular components of key plant hormone signalling pathways that rely on normal functional membranes for their correct function.

Chapter 5

Results:

Signalling in Development: Ethylene

Introduction

The pleiotropic phenotypes of *hydra1* and *hydra2/fackel* suggested that correct sterol profiles within the membranes may be responsible for defects in a number of different signalling pathways. Brassinosteroids do not rescue the phenotype in either *hydra1* or *hydra2/fackel*, however defects in the activities of a number of other major plants hormones could be involved in creating the *hydra* phenotype. The root hair phenotype of *hydra2/fackel* suggests defective ethylene signalling, as ethylene is a positive regulator of hair formation (Tanimoto *et al.*, 1995) and reduces root growth when plants are supplied with ethylene (Johnson and Ecker, 1998). Both *hydra1* and *hydra2/fackel* roots have root hairs closer to the root tip than is seen in the wild type, with very abnormal patterning evident in *hydra2/fackel*. The aim of this chapter is to present the results of a number of experiments designed to investigate the roles of look signalling pathways involving ethylene in contributing to the *hydra* phenotypes.

5.1 *hydra* Root Growth is Rescued by Chemically Inhibiting Ethylene Perception with Silver Ions

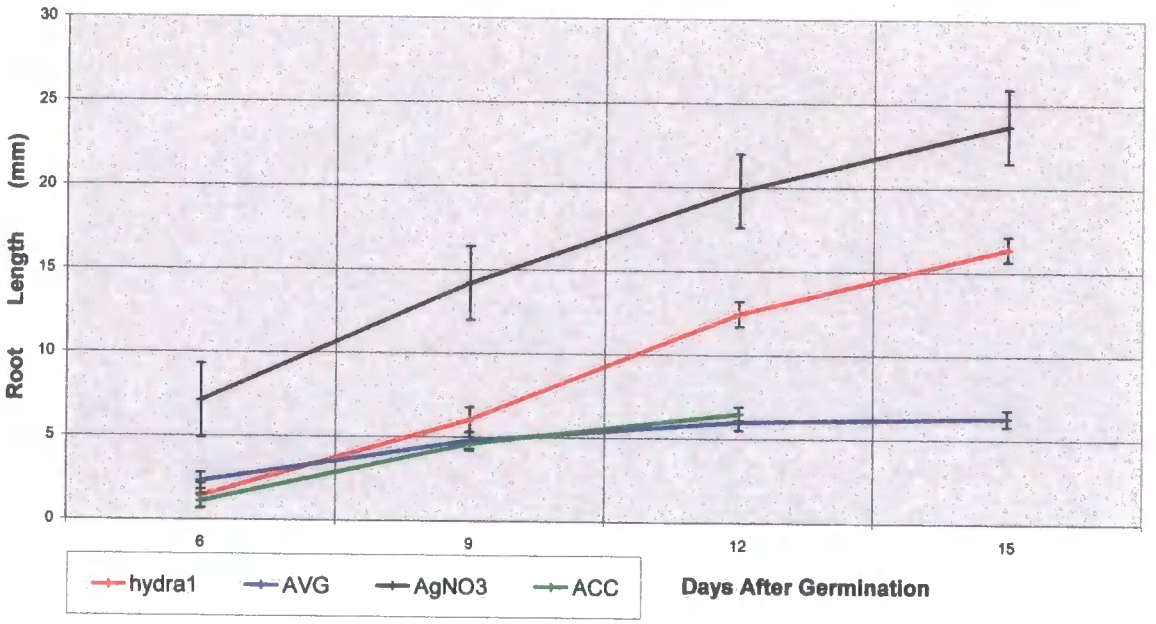
In order to investigate the influence of ethylene on the development and growth of the main root, a number of initial experiments using chemical inhibitors were performed. Seedlings were transferred 3 days after germination onto plates containing half-strength MS medium and one of three compounds. Aminoethoxyvinylglycine (AVG) is an ethylene biosynthesis inhibitor acting on ACC synthase enzymes (Lieberman *et al.*, 1975; Tanimoto *et al.*, 1995). Silver ions, in the form of silver nitrate ($AgNO_3$), inhibit ethylene receptors within the cell membrane by interfering with intramolecular signal propagation within the receptor (Rodriguez *et al.*, 1999). ACC is a precursor for ethylene in plants, supply of which therefore increases the amount of ethylene produced within the plant tissues (Liang *et al.*, 1998).

hydra1

Figure 5.1.1 shows that when grown on 0.5μM AVG, *hydra1* showed reduced rather than increased root growth, suggesting that there is no overproduction of ethylene within this mutant. The reduction in growth rate may be due to AVG toxicity. When grown on 10μM $AgNO_3$ however, *hydra1* exhibited an increase in root growth to wild type levels, suggesting that *hydra1* may exhibit an ethylene perception problem rather

than overproducing ethylene. When supplied with 10μM ACC, there is a reduction in *hydra1* root growth when compared with untreated *hydra1* seedlings, is more comparable with *hydra2* root growth under standard conditions. These result suggests that *hydra1* does not overproduce ethylene and although silver ions can increase root growth, there does not seem to be a problem with ethylene signalling as increasing the levels of ethylene reduces root growth, mimicking *hydra2* root growth under standard conditions.

Figure 5.1.1 Influence of Ethylene on *hydra1* Root Growth

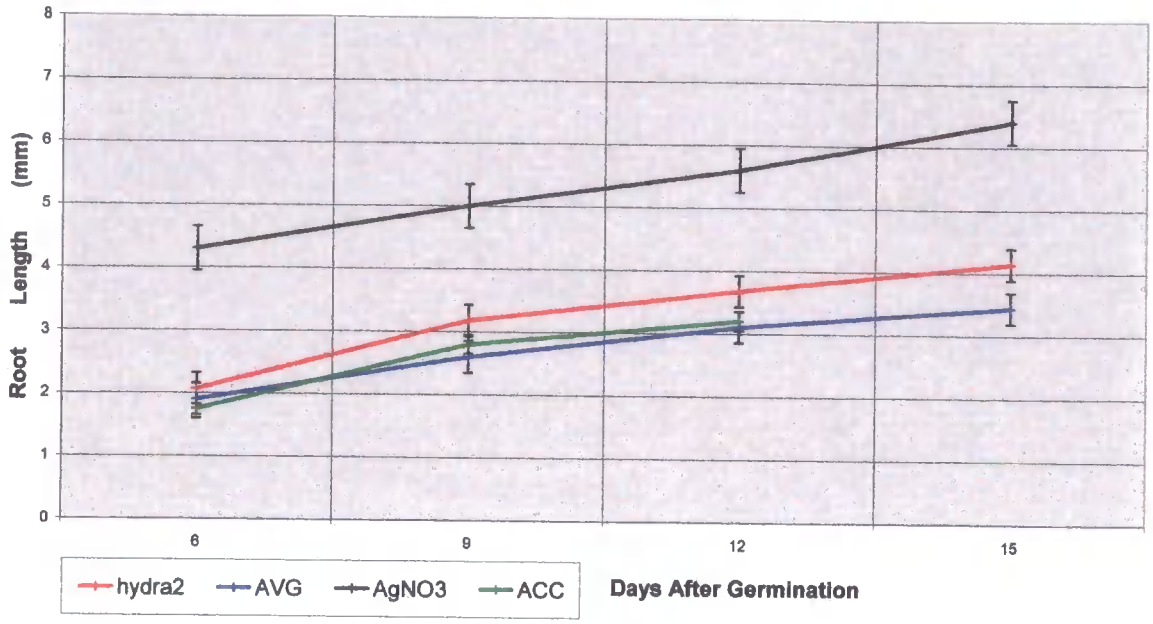


hydra2

Figure 5.1.2 shows that when *hydra2* is grown on 0.5μM AVG it also showed reduced rather than increased root growth, suggesting that there is no overproduction of ethylene within either of the *hydra* mutants. On 10μM $AgNO_3$ *hydra2* root growth was increased, causing an extension to the life of the main root meristem. The main root continued to grow beyond day 15, when in untreated *hydra2* seedlings there is a complete reduction in cell division and loss of the root cap.

Unlike *hydra1*, when *hydra2* was grown on media supplemented with 10μM ACC, there was only a small reduction in root growth, indicating that the addition of more ethylene to *hydra2* seedlings does not cause any further increase in the severity of the root phenotype.

Figure 5.1.2 Influence of Ethylene on *hydra2* Root Growth



Because AVG did not rescue either of the mutants, they are probably not overproducing ethylene; however only direct measurements of ethylene levels will answer this question satisfactorily. Silver ions rescued the root growth of both *hydra1* and *hydra2*, and in *hydra2* this involved a prolonged activity in the meristem beyond day 15. Addition of exogenous ACC caused reduced root growth in both mutants. However in *hydra1* the reduction caused a *hydra2* phenocopy, whilst *hydra2* root growth was reduced only fractionally. Together these results suggest that ethylene signalling is abnormal, and that these abnormalities are associated with the ethylene receptor rather than because of an overproduction of ethylene.

5.2 Silver Ions Rescue *CYCAT1:CDB:GUS* Expression in the Main Root of *hydra2*

When the *hydra2CYCAT1:CDB:GUS* line was grown under standard conditions expression of the cell division marker diminished from day 12 and had disappeared by day 18 (Figure 3.15). Following from the observation made above, *hydra2CYCAT1: CDB:GUS* seedlings were transferred to media containing 10μM $AgNO_3$ or 10μM ACC 6 days after germination, and allowed to grow on the surface of the agar and then stained for GUS activity on day 18.

Figure 5.2

Rescue of *CYCAT1:CDB:GUS* Expression in the *hydra2* Root Meristem

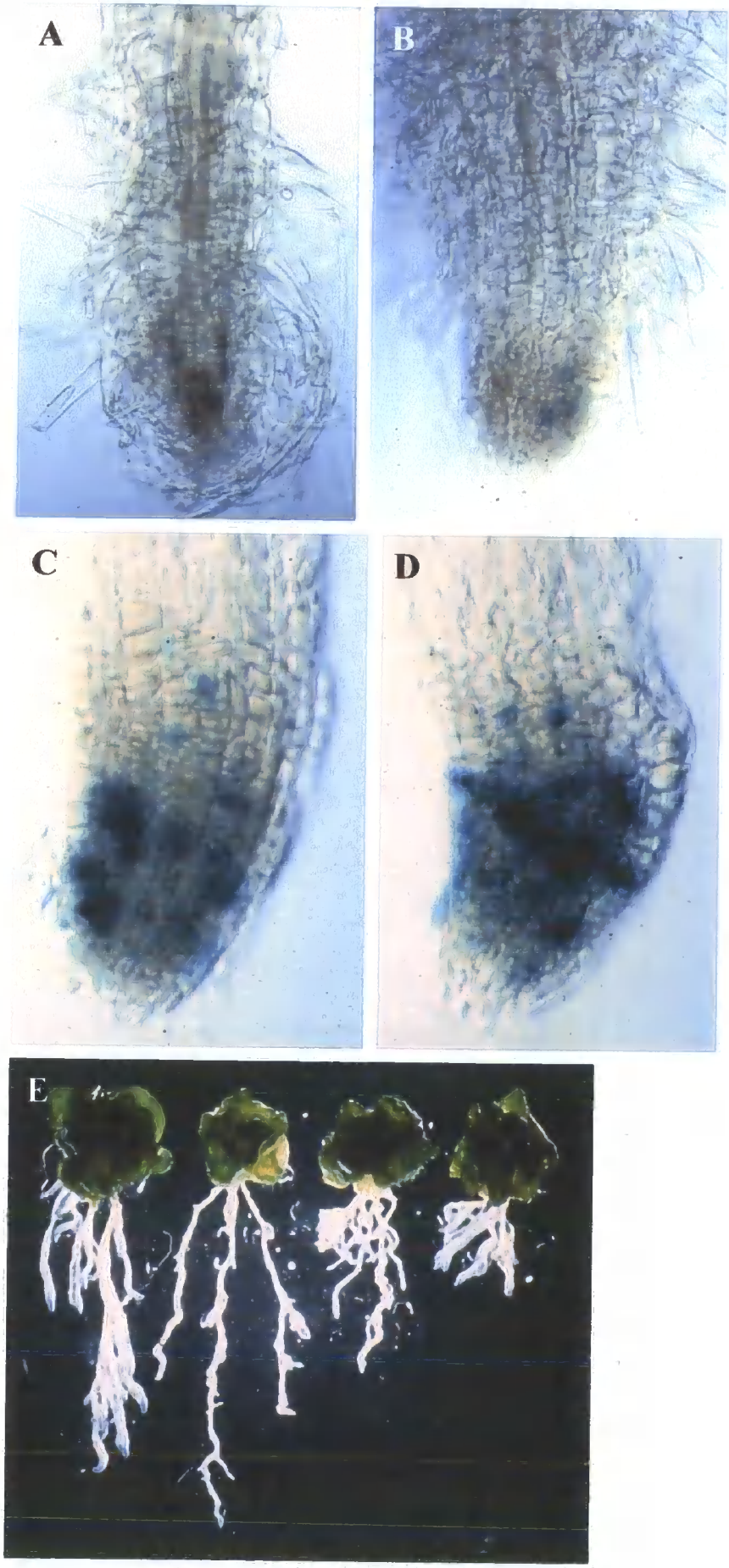
A and B: *hydra2CYCAT* seedlings transferred 6 DAG onto medium supplemented with 10μM ACC, 18 DAG (mag. x 20).

C and D: *hydra2CYCAT* seedlings transferred 6 DAG onto medium supplemented with 10μM $AgNO_3$, 18 DAG (mag. x 30).

Enhanced Rescue of Cell Division by Silver Ions in the *hydra2exo* Double Mutant

E: (L-R) *hydra2exo/exo*, *hydra2exo/exo* transferred 6 DAG onto medium supplemented with 10μM $AgNO_3$, *hydra2 EXO/exo* transferred 6 DAG onto medium supplemented with 10μM $AgNO_3$, and *hydra2 EXO/exo*, 21 DAG.

Figure 5.2



By 18 days after germination GUS activity of the cyclin reporter was absent from the primary root meristem of untreated *hydra2* seedlings, demonstrating a loss of cell division activity. Supplying ACC caused only a small reduction in root growth, as mentioned above, and consistent with this there was also no detectable GUS staining within the primary root of seedlings grown on 10μM ACC 18 days after germination (Fig. 5.2, A and B).

When seedlings were grown on 10μM $AgNO_3$, which inhibits ethylene perception at the receptor, GUS activity was recovered and the expression pattern was wild type in appearance (Fig. 5.2, C and D). These results link defective ethylene signalling within the *hydra2* root with cell division activity within the meristem.

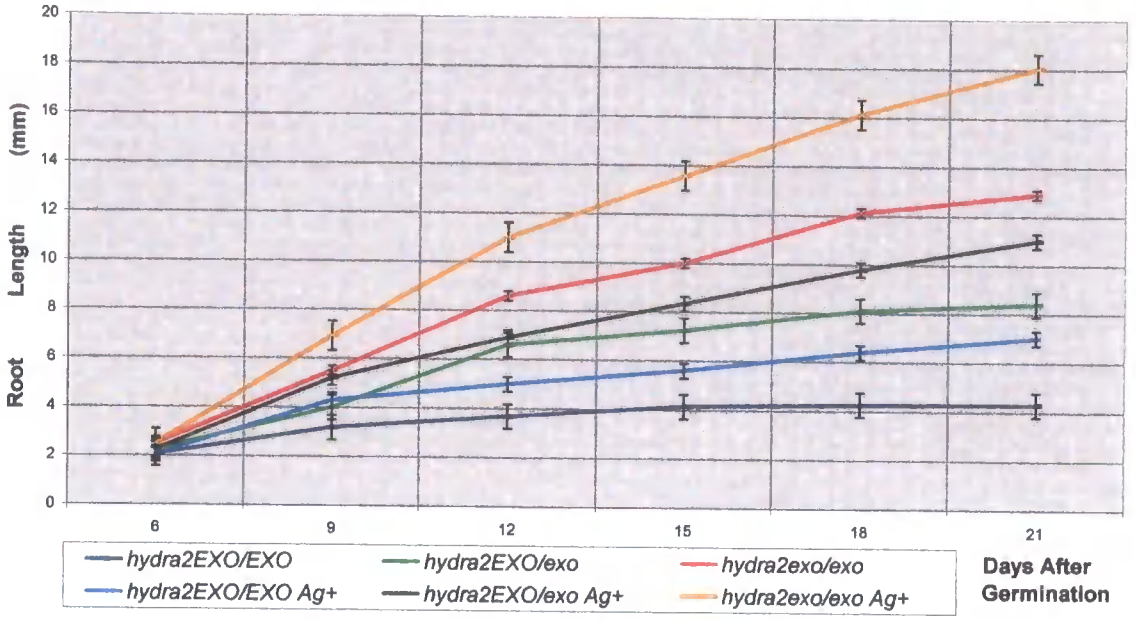
5.3 Mutations in the *EXORDIUM* Gene Enhance the Rescue of the *hydra2* Root Meristem by Silver Ions

As shown in section 3.16, mutations in the *EXORDIUM* gene rescue root growth and prolong the activity of the primary root meristem in *hydra2*. In view of the rescue of root growth by silver ions, 32 F2 *hydra2exordium* double mutant seedlings were transferred to plates containing 10μM $AgNO_3$ 6 days after germination, their primary roots were measured every three days and then all of the plants were individually GUS stained on day 21. Root lengths were then plotted and averaged depending on the segregating population, as described in section 3.16.

The *exordium* mutation showed a heterozygous phenotype in the *hydra2* background, *hydra2* seedlings homozygous for the *exordium* mutation had longer roots than *hydra2* mutants heterozygous for the *exordium* mutation (section 3.16). When grown in the presence of 10μM $AgNO_3$, both the predicted heterozygous (16 out of 32 seedlings) and homozygous (8 out of 32 seedlings) *exordium* double mutants showed an increase in the ability of silver ions to enhance the amount of root growth.

Figure 5.3 shows a comparison in the root growth of treated and untreated seedlings in the three segregating populations, and which is also seen in Figure 5.2, image E.

Figure 5.3 *hydra2exo* Double Mutants Grown With Silver Ions



These results show that the rescue of *hydra2* root growth by silver ions can be enhanced when the *exordium* mutation is present in the *hydra2* background.

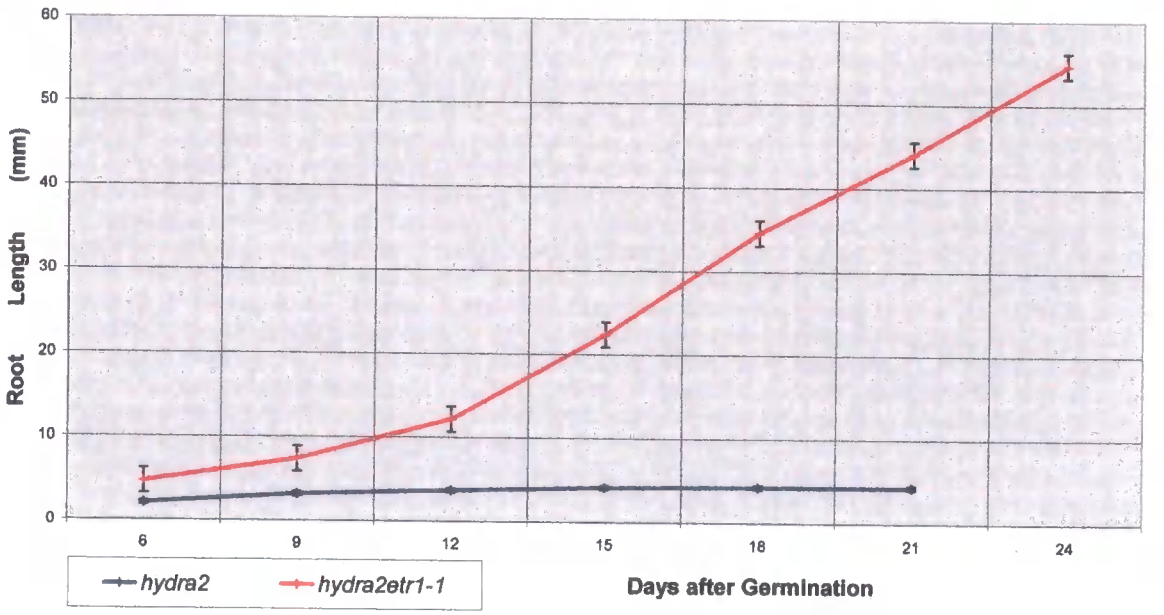
5.4 Mutations in the Ethylene Receptor *ETR1* in *hydra2* Copies the Pharmacological Rescue of the Primary Root by Silver Ions

To further investigate the ethylene receptor, the site at which the silver ions inhibit ethylene perception, a genetic approach was taken to complement the pharmacological approach used thus far. *hydra2* mutants were crossed with the *ethylene resistant1* (*etr1-1*) mutant, defective in a member of the ethylene receptor family (Chang *et al.*, 1993). *etr1-1* is the only allele that binds ethylene but does not propagate a signal (Hall *et al.*, 1999). *etr1-1* mutants look wild type in appearance, but have longer roots that are resistant to inhibition by ACC. Putative *hydra2etr1-1* double mutants were identified from F2 populations that contained wild type seedlings that had longer roots in the presence of ACC, i.e. contained *etr1-1* mutants within the population. *hydra*-like seedlings were then subjected to root growth assays under standard conditions so that the segregation ratios could be worked out.

18 out of 60 (approximately 1/4) seedlings showed a root length that was longer than *hydra2*. This population represents the predicted double homozygous mutants, and

indicated that only *hydra2* seedlings homozygous for the *etr1-1* mutation had longer roots. Figure 5.4.1 shows that the amount of root growth in the double mutant was considerably increased, on day 21 root growth was 11-fold higher in the double mutant (44.05 ± 2.64) than in *hydra2* (4.29 ± 0.18).

Figure 5.4.1 *hydra2etr1-1* Root Growth



The rescue of *hydra2* root growth by *etr1-1* was clearer than chemically inhibiting the action of the receptor with silver ions.

Further analysis revealed that the *hydra2etr1-1* double mutants showed a rescue of more than just root growth. Figure 5.4.2 (A and B) shows seedlings of wild type, *etr1-1*, *hydra2* and the *hydra2etr1-1* double mutants. When root hair patterning in 9 day old *hydra2etr1-1* seedlings was viewed under the a dissecting microscope, there was a clear rescue of the patterning (Fig. 5.4.2, G, H and I). The ectopic branched root hairs of *hydra2* (Fig. 5.4.2, D and I) were rescued to a spacing and specification similar to that of wild type (Fig. 5.4.2, H and F), with single root hairs developing from the correct cell files, and initiating more proximal to the root tip than in *hydra2*.

When *hydra2* roots were stained with lugol 10 days after germination (Fig. 5.4.2, J) they showed the presence of a columella root cap on the main root. However by day 14 staining was absent, indicating the loss of the root cap (Fig. 5.4.2, K) (Section 3.13). When *hydra2etr1-1* double mutants were stained 14 days after germination not only was

Figure 5.4.2

***hydra2etr1-1* Double Mutants**

A: (L-R) wild type, *etr1-1*, *hydra2etr1-1* and *hydra2* seedlings for comparison of root length, 12 DAG.

B: (L-R) two *hydra2etr1-1* double mutants and two *hydra2* mutants, 12 DAG.

C and D: *hydra2*, 12 DAG (C mag. x 6, D x 10).

E and F: *hydra2etr1-1* double mutant, 12 DAG (E mag. x 6, F x 10).

G: 9 day old wild type root hairs (mag. x 10).

H: 9 day old *hydra2etr1-1* double mutant root hairs (mag. x 10).

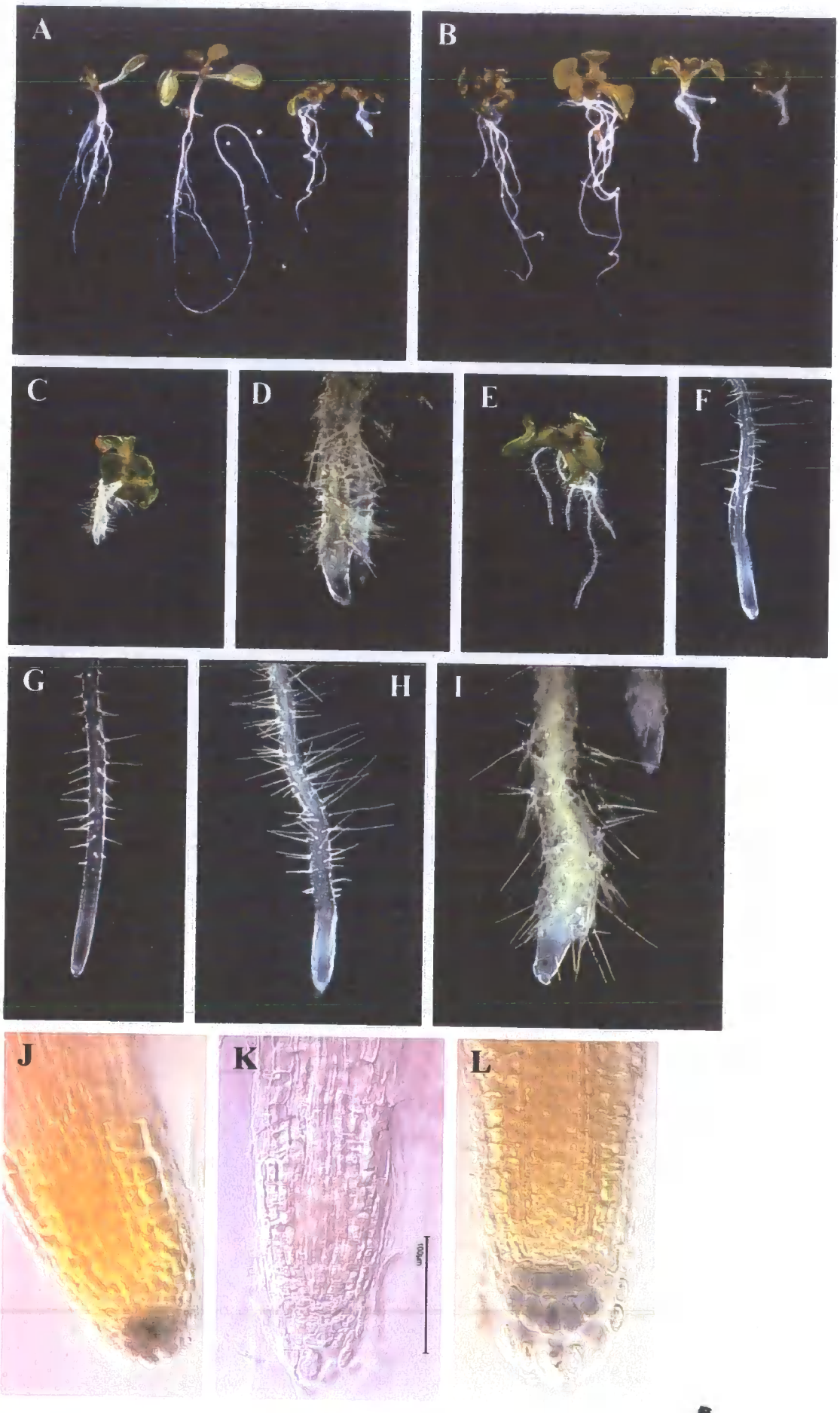
I: 9 day old *hydra2* seedling root hairs (mag. x 10).

J: 10 day old *hydra2* primary root stained with lugol (mag. x 30).

K: 14 days old *hydra2* primary root stained with lugol (mag. x 30).

L: 14 day old *hydra2etr1-1* double mutant primary root stained with lugol (mag. x 30).

Figure 5.4.2



the root cap columella still present, but the root tip showed more normal patterning (Fig. 5.4.2, L).

In the shoot there appeared to be some rescue of the length of the petiole in *hydra2etr1-1* double mutants, both root and petiole elongation are more evident in the *etr1-1* double mutant compared with *hydra2*.

These results indicate that there is defective ethylene signalling in the *hydra2* mutant.

5.5 Mutations in *ein2* Cause Total Ethylene Insensitivity and Rescue Both Shoot and Root Growth in the *hydra* Mutants

ETR1 is predominantly expressed in the root and yet some phenotype rescue of the shoot was seen in the *hydra2etr1-1* double mutant. In order to see if any further phenotypic characteristics could be rescued by inhibiting ethylene perception, a second ethylene signalling mutant was selected for crossing. *ETHYLENE INSENSITIVE2* (*EIN2*) represents a membrane-bound component of the ethylene signalling pathway that is downstream of the receptor family. *ein2* mutants are completely resistant to ethylene; *EIN2* is the only homologue of this component in *Arabidopsis* (Alonso *et al.*, 1999).

Double mutants were identified within F2 populations containing wild type seedlings that were insensitive to ACC, i.e. were *ein2* mutants.

hydra1

31 F2 *hydra*-like seedlings were grown on vertical agar plates under standard conditions, and the main root was measured every three days. 9 out of 31 seedlings (approximately 1/4) showed a longer root phenotype, representing the predicted *hydra1ein2* double homozygous population and indicating that there is no phenotype in the root of heterozygous *ein2 hydra1* mutants. Figure 5.5.1 shows the increase in root growth seen in the homozygous *hydra1ein2* double mutants when compared with *hydra1*.

Figure 5.5.1 *hydra1ein2* Root Growth

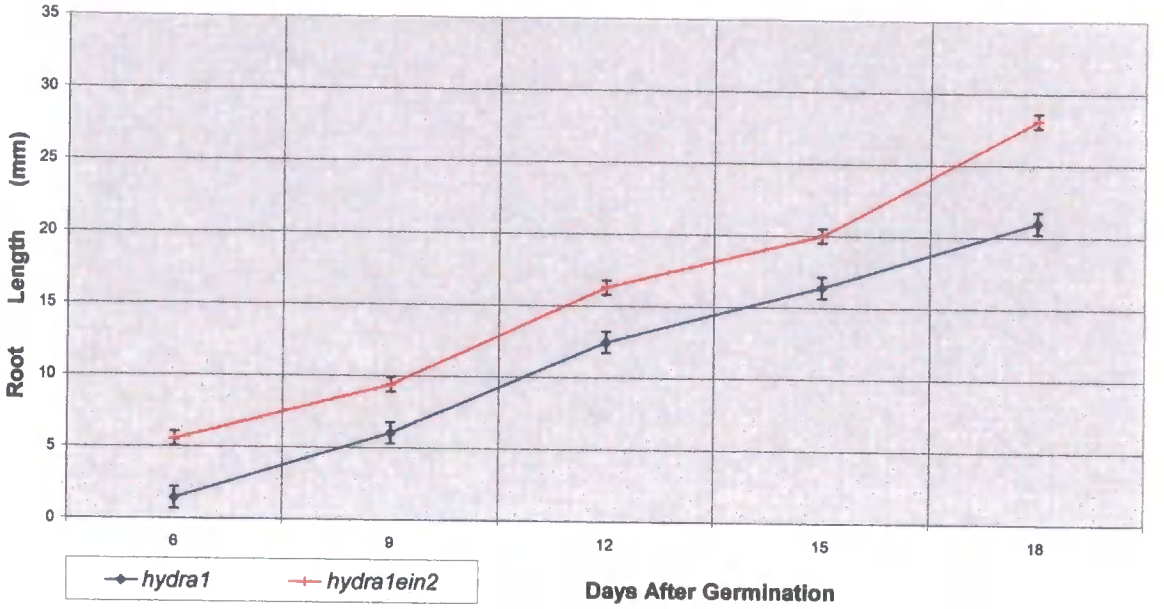


Figure 5.5.2 shows a comparison of the shoot of the *hydra1ein2* double mutants with *hydra1* mutants. As well as the difference in root length (A), there was also an increase in the amount of shoot growth (Fig. 5.5.2, A and D, B and E). The petioles of the cotyledons and leaves were elongated (Fig. 5.5.2, F and H), and the hypocotyl was more elongated and thinner than in *hydra1* (Fig. 5.5.2, C). Upon closer inspection of the leaves it was clear that there was a more organised vascular pattern in the double mutants (Fig. 5.5.2, I, J and K). The petiole and blade of the leaf had expanded more than normal, and the vascular strand entering the leaf was not duplicated. The primary, secondary and tertiary vascular strands were present and there were no isolated vascular elements.

hydra2

23 out of 31 F2 *hydra*-like seedlings (approximately 3/4) grew longer roots than *hydra2* mutants, indicating that in *hydra2* the *ein2* mutation had an effect on root growth in the heterozygous state. Three segregating populations could be discerned, representing *hydra2* (8 individuals), *hydra2* mutants heterozygous for the *ein2* mutation (16 seedlings), and *hydra2* mutants homozygous for the *ein2* mutation (7 seedlings). Figure 5.5.3 shows the root growth of these three predicted segregating populations.

Figure 5.5.2

***hydra1ein2* Double Mutants**

A: *hydra1* and a *hydra1ein2* double homozygous mutant.

B and C: The shoot region of *hydra1ein2* double mutants, note the recovery of the hypocotyl in C.

D: A comparison of the shoot region of *hydra1* and *hydra1ein2*.

E: 18 day old *hydra1ein2* double mutant shoot.

F: A comparison of the cotyledons, *hydra1ein2* on top, *hydra1* below (mag. x 5).

G and H: A comparison of the leaves, *hydra1ein2* on top, *hydra1* below (mag. x 5).

I: A *hydra1* leaf (mag. x 10).

J and K: A *hydra1ein2* double mutant leaf (mag. x 10).

All seedlings 15 days after germination.

Figure 5.5.2

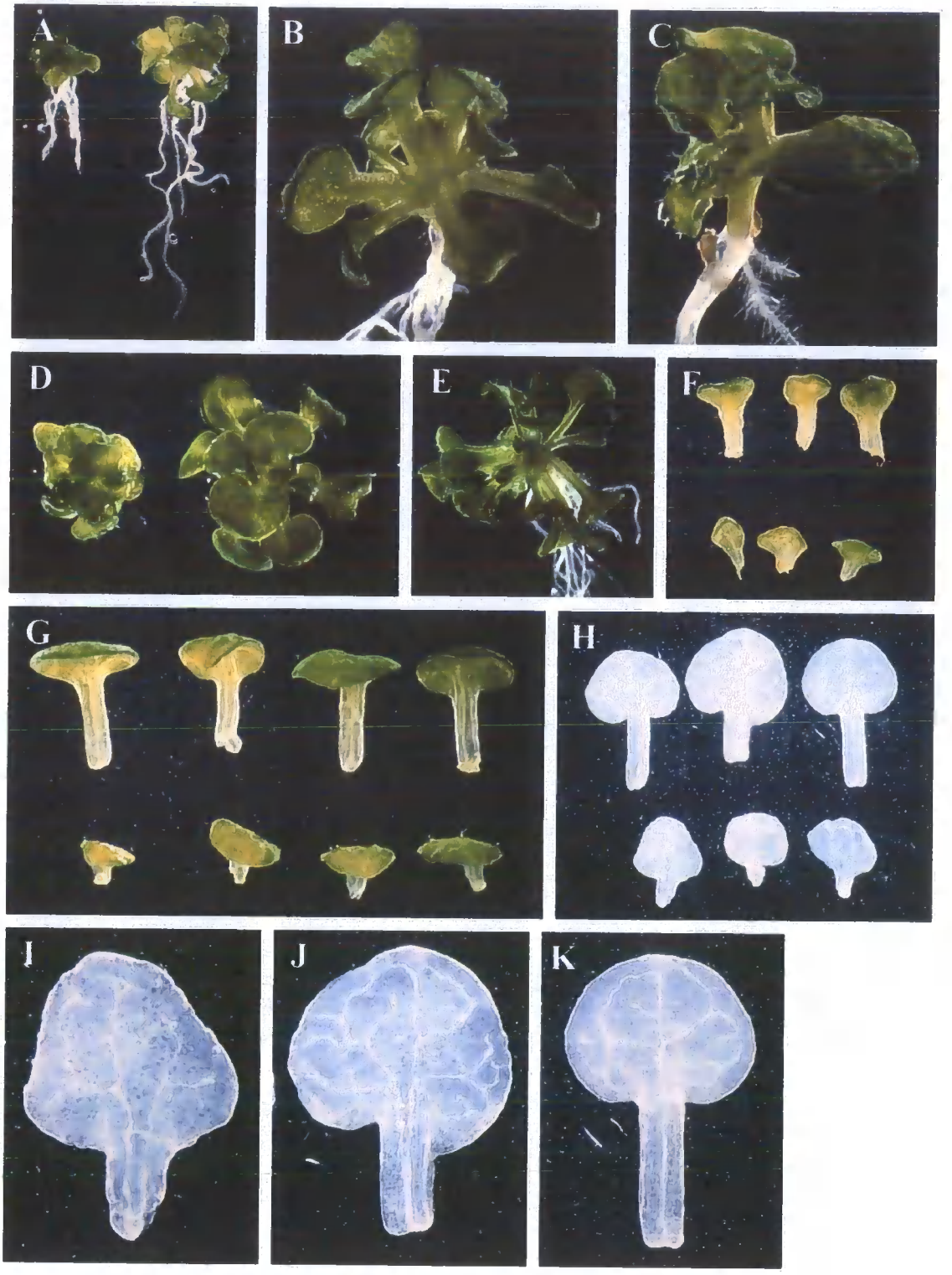
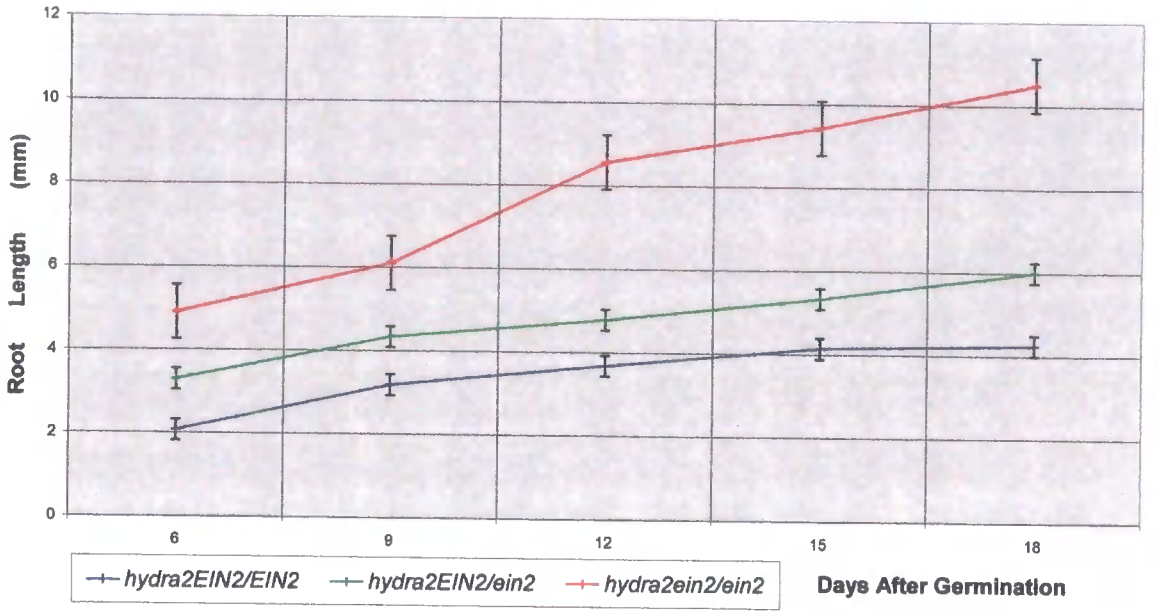


Figure 5.5.3 *hydra2ein2* Root Growth



hydra2ein2/ein2 (18 days, 10.5 ± 0.88 mm) mutants had considerably longer roots than *hydra2EIN2/ein2* (18 days, 6.0 ± 0.31 mm) mutants, which had only slightly longer roots than normal *hydra2* mutants (18 days, 4.25 ± 0.19 mm). This indicated that the *hydra2* root meristem was sensitive to the level of ethylene signal transduction, and that having only half the functional amount of *EIN2* was enough to increase root growth when compared with fully ethylene sensitive plants. The *hydra2* root meristem responded differently to exogenous ethylene and ethylene inhibitors when compared with *hydra1*, and the effect of the *ein2* mutation on the root meristems of the two mutants confirms these differences.

Figure 5.5.4 shows the phenotype of the *hydra2ein2* double homozygous mutants. The shoot of *hydra2ein2/ein2* mutants was not rescued to the same extent as the *hydra1* shoot, however, there appeared to be more of an effect on the cotyledons in *hydra2* than on the leaves (Fig. 5.5.4, A and B). The blade of the leaves was more expanded than normal (Fig. 5.5.4, D), however there was less elongation of the petiole than seen in the *hydra1ein2* double mutants. The vasculature of the leaves also had a more wild type pattern; the primary, secondary and tertiary vascular strands were present and there were no isolated vascular elements. The petioles of the cotyledons had elongated in the double homozygous mutants (Fig. 5.5.4, E), and the vascular patterning was more organised and not duplicated or discontinuous (Fig. 5.5.4, G and H).

Figure 5.5.4

***hydra2ein2* Double Mutants**

A: Comparison of the shoot region of *hydra2*.

B: Comparison of the shoot region of *hydra2ein2* double homozygote.

C: A *hydra2ein2* homozygote and *hydra2*.

D: Comparison of the leaves, *hydra2ein2* above, *hydra2* below (mag. x 5).

E: A comparison of the cotyledons, *hydra2ein2* above, *hydra2* below (mag. x 5).

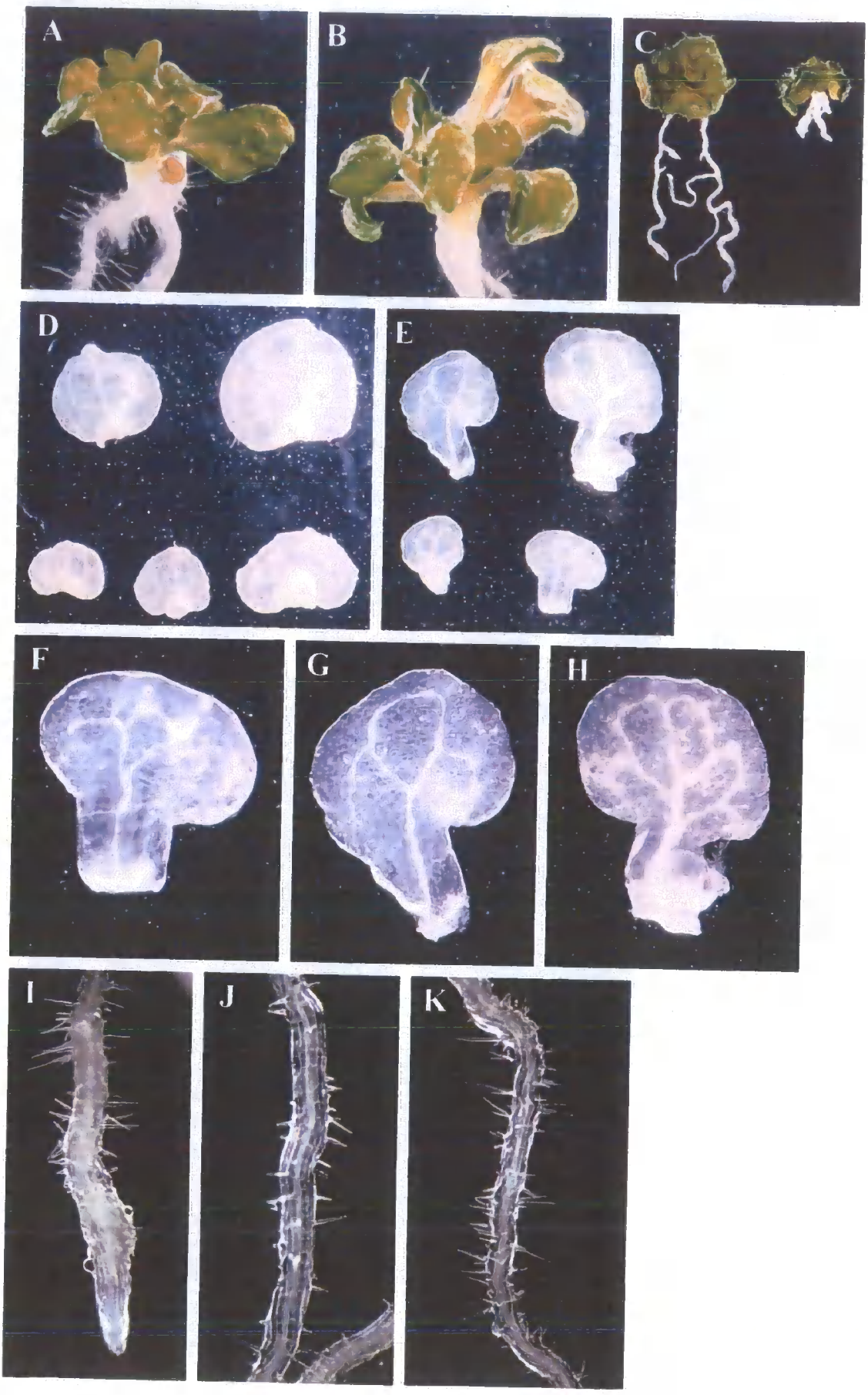
F: The vascular pattern in a *hydra2* cotyledon (mag. x 12).

G and H: The patterning of *hydra2ein2* cotyledons (mag. x 12).

I, J and K: Development of root hairs on *hydra2ein2* double mutants (mag. x 12).

All seedlings 15 days after germination.

Figure 5.5.4



Microscopic analysis of the root epidermis showed that the initiation and development of root hairs was considerably reduced when compared to normal *hydra2* mutants (Fig. 5.5.4, I, J and K). Single short root hairs were initiating from the epidermis, giving the root the a stubbly appearance, however there were less hairs initiating than in *hydra2* mutants. These results indicate that defects in ethylene signalling are not only influencing the activity of the root meristem, but also the specification and growth of root hairs in the *hydra2* mutants. The *ein2* mutation causes complete ethylene insensitivity whilst the *etr1-1* mutation only reduces the amount of ethylene perception, and predominantly within the root. We can conclude that ethylene has a major effect on the *hydra2* root phenotype, as well as an effect upon the development of the shoot.

5.6 *ACC SYNTHASE1::GUS* Expression is Abnormal in *hydra2*

Some members of the ACC synthase gene family are specifically responsive to hormones. Both auxin and cytokinin have been implicated in acting through ethylene and regulating the levels of ACC synthase enzymes, the rate limiting enzyme in the ethylene biosynthetic pathway (Liang *et al.*, 1995). Ethylene also feeds back on its own biosynthetic enzymes so as to be able to allow a level of self-regulation.

ACS1 is a pseudo ACC synthase, lacking essential residues for catalysis of the conversion of *S*-adenosyl methionine to ACC (Liang *et al.*, 1995). However the *ACS1::GUS* line exhibits both developmental and hormonal regulation (Rodrigues-Pousada *et al.*, 1999). Such a gene therefore acts as a marker to allow an understanding of the internal balance of different hormones within specific tissue at specific time points in development, and as such offered a useful tool for investigating the *hydra* mutants. *ACS1* expression in the root is repressed by auxin but activated by cytokinin. Ethylene also increases the expression of this gene, indicating a positive feedback mechanism (Rodrigues-Pousada *et al.*, 1999).

ACS1::GUS (courtesy of Dominique Van Der Straeten, Gent University) was therefore crossed into both *hydra* backgrounds. GUS activity in *hydra* mutants containing the *ACS1::GUS* construct was localised by histochemistry throughout development, up to 21 days post-germination.

Figure 5.6

Developmental Expression of *ACS1::GUS* in the Primary Root of *hydra2*

A to E: *ACS1::GUS* expression in wild type (various ages) (A and C mag. x 15, B x 8, D x 20, E x 40).

Expression is restricted to the endodermal and vascular tissues from the hypocotyl root junction to the zone of differentiation in the root tip. No expression is seen distal to the zone of elongation in the root tip. There is no change in the expression pattern in the root over time.

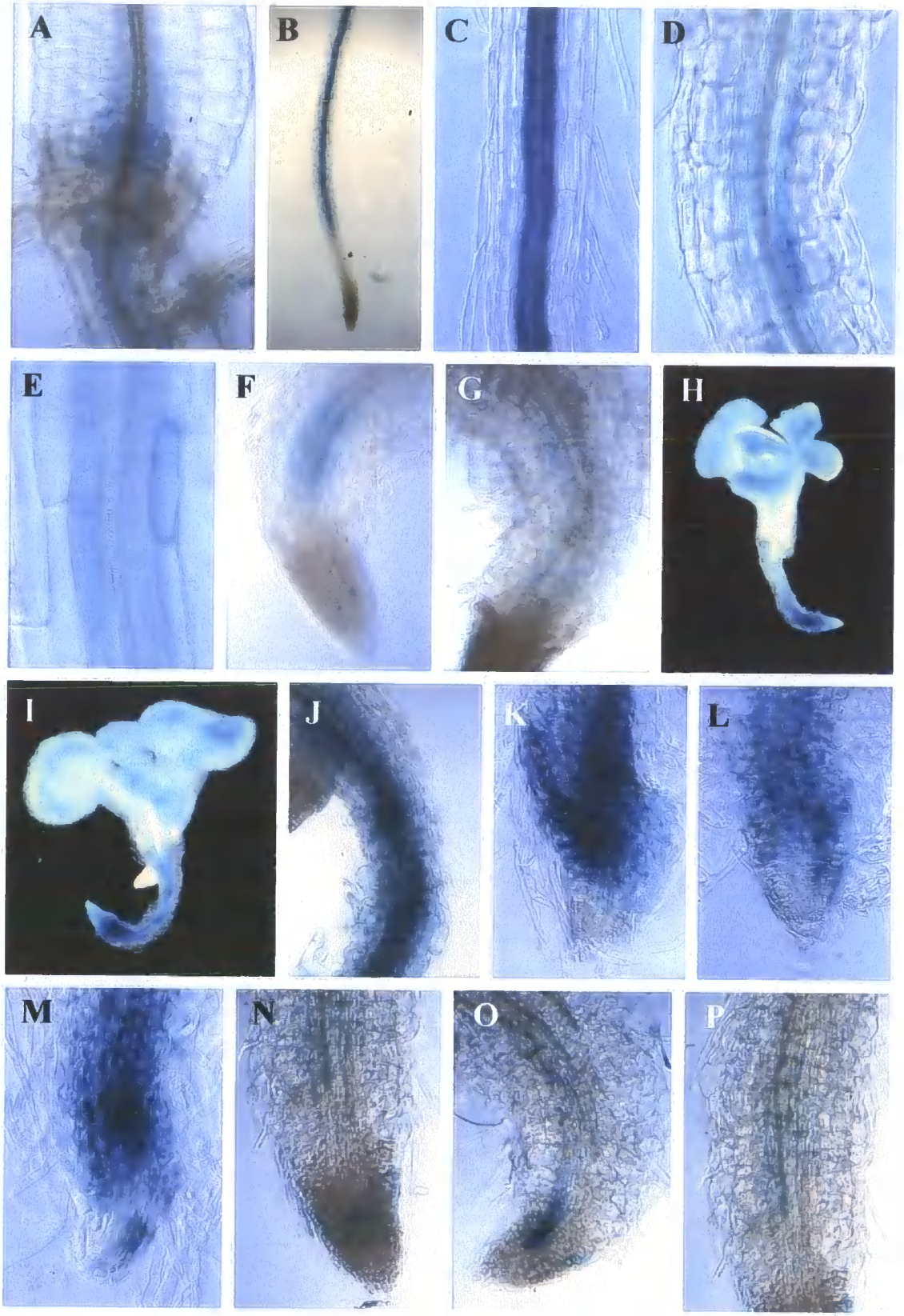
F to P: *ACS1::GUS* expression in *hydra2* (F, G, J, K, L, M, N, O and P mag. x 20, H and I x 5).

F and G show 3 day old seedlings.

H to M show 6 day old seedlings.

N to P show 9 day old seedlings.

Figure 5.6



ACS1::GUS expression in a wild type genetic background was restricted to the endodermal and vascular tissues from the hypocotyl root junction to the zone of differentiation in the root tip. No expression was seen distal to the zone of elongation in the root tip (Figure 5.6, A to E).

In a *hydra2* background, *ACS1::GUS* expression was the same as wild type in the primary root (3 DAG, Fig. 5.6, F and G) until 6 days post-germination, when there was a strong increase in GUS activity throughout the entire root - in all radial layers and even extending into the meristem and the distal root tip (Fig. 5.6, H to M). By day 9, however this ectopic expression was down-regulated to the same level as that seen on day 3 (Fig. 5.6, N, O and P), and was unchanged thereafter (pictures not shown). By contrast *hydra1* showed a wild type staining pattern consistently throughout development (pictures not shown).

These results further strengthen the view that the root phenotypes of *hydra1* and *hydra2* are different, and suggest that the ectopic up-regulation of *ACS1::GUS* in *hydra2* may be caused by either defective ethylene or cytokinin signalling.

5.7 Cytokinin not Ethylene is Responsible for the Ectopic Expression of *ACS1::GUS* in *hydra2*

To test whether the ectopic up-regulation of GUS in the *hydra2* mutants was due to high levels of ethylene or cytokinin, *ACS1::GUS* and *hydra2ACS1::GUS* seedlings were transferred to media containing either 10μM ACC or 10μM BA 3 days post germination, and grown for six days before histochemical staining.

Consistent with the observations made by Rodrigues-Pousada *et al.*, (1999), *ACS1::GUS* was up-regulated by cytokinin and ethylene (Figure 5.7, A, B and C). However, this hormonal regulation was not the same in the *hydra2* background. ACC did not increase the expression of *ACS1::GUS*, indeed it reduced the level of expression of the gene (Fig. 5.7, D to G). In contrast, and consistent with Rodrigues-Pousada *et al.*, (1999), cytokinin (BA) did increase the expression of the reporter (Fig. 5.7, H, I and J), and it did so in a way that mimicked the expression seen in 6 day old *hydra2* seedlings grown under standard conditions.

Figure 5.7

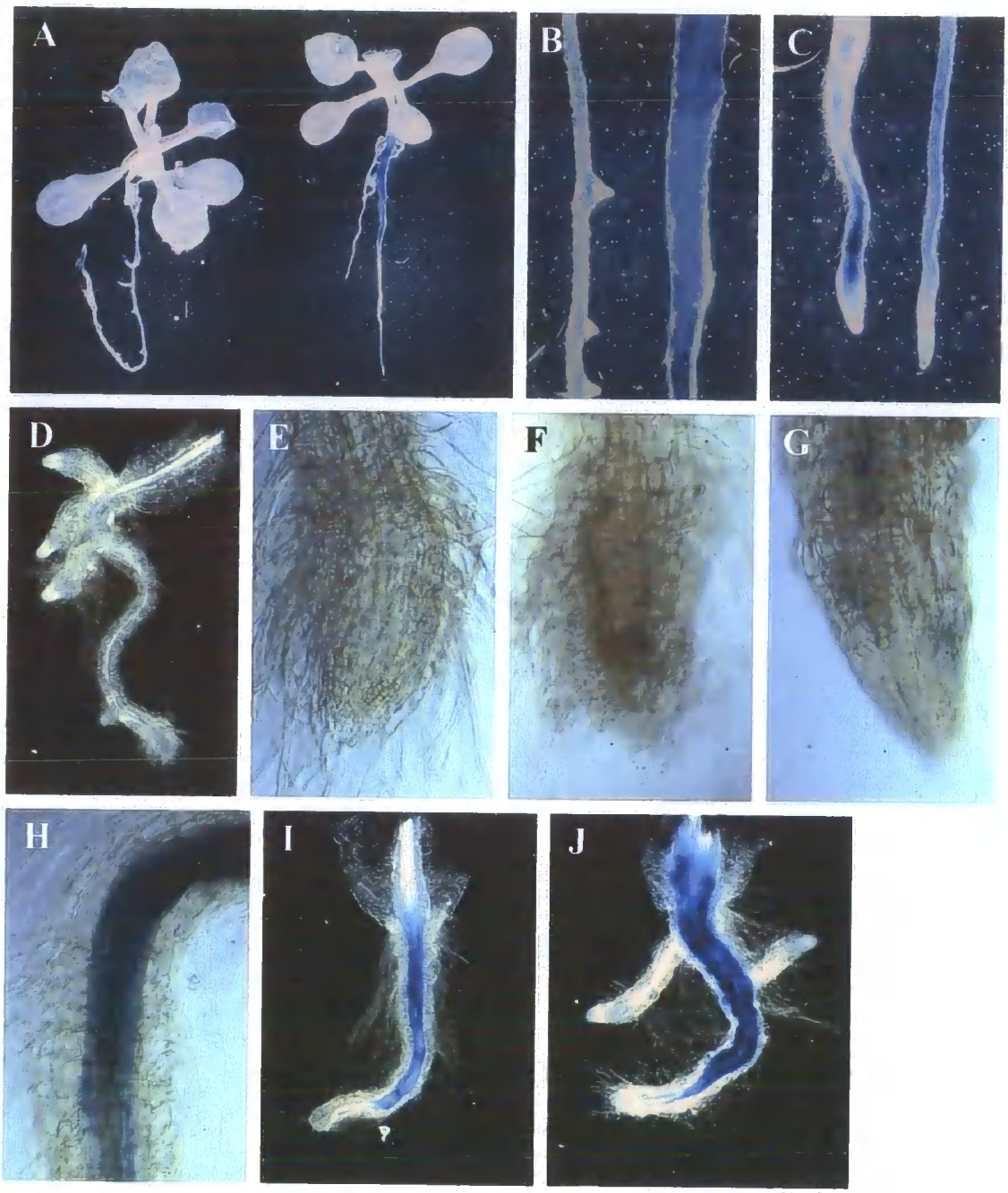
***ACS1::GUS* Expression in Response to Ethylene and Cytokinin in *hydra2* Seedlings**

A to C: *ACS1::GUS* expression in 12 day old wild type seedlings transferred onto (L-R) either 10μM ACC or 10μM BA 6 days after germination.

D to G: *ACS1::GUS* expression in 12 day old *hydra2* seedlings transferred onto 10μM ACC 6 days after germination (D mag. x 8, E, F and G x 20).

H to J: *ACS1::GUS* expression in 12 day old *hydra2* seedlings transferred onto 10μM BA 6 days after germination (H mag. x 20, I and J x 8).

Figure 5.7



These results suggest that cytokinin induces the observed ectopic expression of *ACS1::GUS* on day 6 in *hydra2*, and that ethylene signalling is disrupted, such that the self regulation of its own biosynthetic enzymes does not occur.

5.8 Conclusions

Evidence has been presented in this chapter showing that ethylene signalling within the *hydra* mutants is defective, possibly due to constitutive activation of the pathway rather than through overproduction of ethylene.

By inhibiting the activity of the ethylene receptor with silver ions or genetically, defective cell division in the root meristem of *hydra2* can be rescued, as well the patterning of the root cap and root hairs. Rescue of defects in aerial parts of the *hydra2* seedling was seen when total ethylene insensitivity was achieved through double mutant analysis with the *ein2* mutant.

Finally, it was shown that cytokinin could be responsible for the ectopic up-regulation of the *ACS1::GUS* reporter gene seen on day 6 in *hydra2*. Ethylene was unable to self-regulate the expression of the reporter, possibly due to the constitutive perception of ethylene in the *hydra2* mutant.

Defective ethylene perception can explain certain elements of the mutant phenotype. In the next chapter more evidence of defects in the signalling pathways of another major plant hormone will be presented.

Chapter 6

Results:

Signalling in Development: Auxin

Introduction

Results presented in the previous chapter suggested that ethylene signalling, and possibly also cytokinin signalling are defective in the *hydra* mutants. A number of phenotypic defects seen in the *hydra* mutants are also suggestive of possible defects in either auxin transport or response. These include the positioning of the cotyledons at the globular stage of embryogenesis and vascular patterning, which each require correct auxin transport (Liu *et al.*, 1996; Mattsson *et al.* 1999). Furthermore, auxin response is crucial in patterning the root meristem (Sabatini *et al.*, 2000). This chapter will present the results of experiments designed to investigate auxin signalling, by the analysis of cellular components of auxin transport and the auxin response pathway in the *hydra* mutants.

6.1 The *hydras* Show Abnormal Responses to Exogenously Applied Auxins

In order to assess the responses of the *hydra* mutants to auxins, preliminary experiments with exogenously applied auxins were conducted. *hydra* seedlings were grown in the presence of 1μM NAA or 0.1μM or 1μM 2,4-D. NAA is a synthetic auxin analogue that is able to pass through the cell membrane and so does not require the influx carrier (Delbarre *et al.*, 1996). 2,4-D is also a synthetic auxin analogue, but one that is not highly membrane permeable and requires the auxin influx carrier in order to enter the cell at high efficiency (Delbarre *et al.*, 1996).

Seedlings were germinated on half-strength MS medium and then transferred to auxin-supplemented medium ca. 6 days post germination. Figure 6.1.1 shows the effects of growing wild type and *hydra* seedlings in the presence of 1μM NAA. Wild type seedlings responded by producing many lateral roots and root hairs (Fig. 6.1.1, B and E). Both *hydra* mutants, however, responded by undergoing a callusing response within 3-5 days of transfer onto the NAA-supplemented media (Fig. 6.1.1, C and F).

When supplied with either 0.1μM or 1μM 2,4-D, as Figure 6.1.2 shows, wild type seedlings produced a large number of lateral roots, the higher concentration generating more lateral roots than did the lower concentration (Fig. 6.1.2, B and E).

Figure 6.1.1

Responses of the *hydras* to NAA

Seedlings were transferred to 1μM NAA supplemented medium 6 days after germination, and grown for a further 5 days.

A: (L-R) *hydra1* and C24.

B: C24 (mag. x 8).

C: *hydra1* (mag. x 6).

D: (L-R) WS and *hydra2*.

E: WS (mag. x 10).

F: *hydra2* (mag. x 6).

Figure 6.1.1

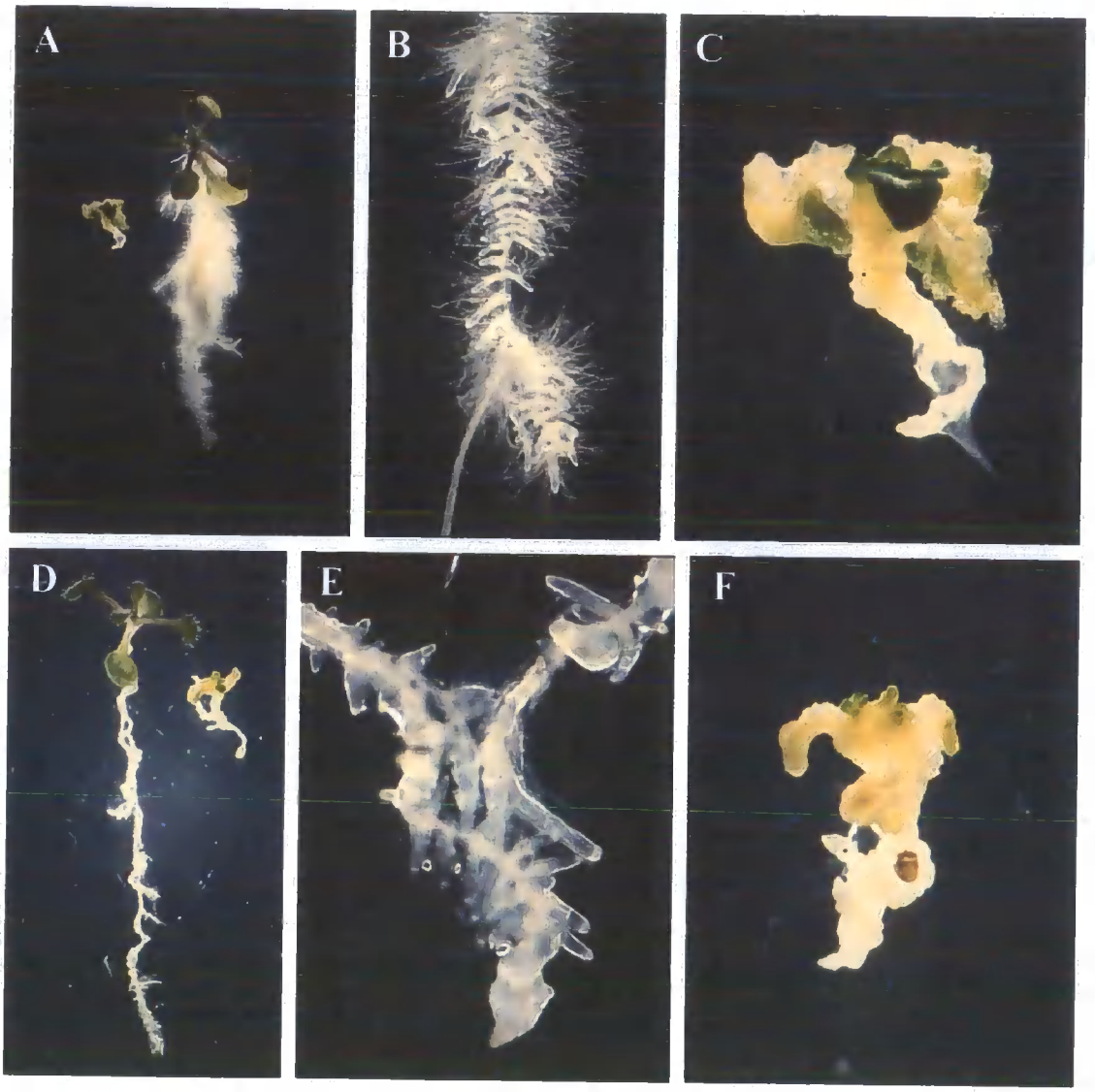


Figure 6.1.2

Response of the *hydras* to 2,4-D

Seedlings were transferred to either 1μM (A to F) or 100nM (G) 2,4-D supplemented medium 6 days after germination, and grown for a further 5 days.

A: (L-R) C24 and 3 *hydra1* seedlings.

B: C24 (mag. x 8).

C: *hydra1* (mag. x 8).

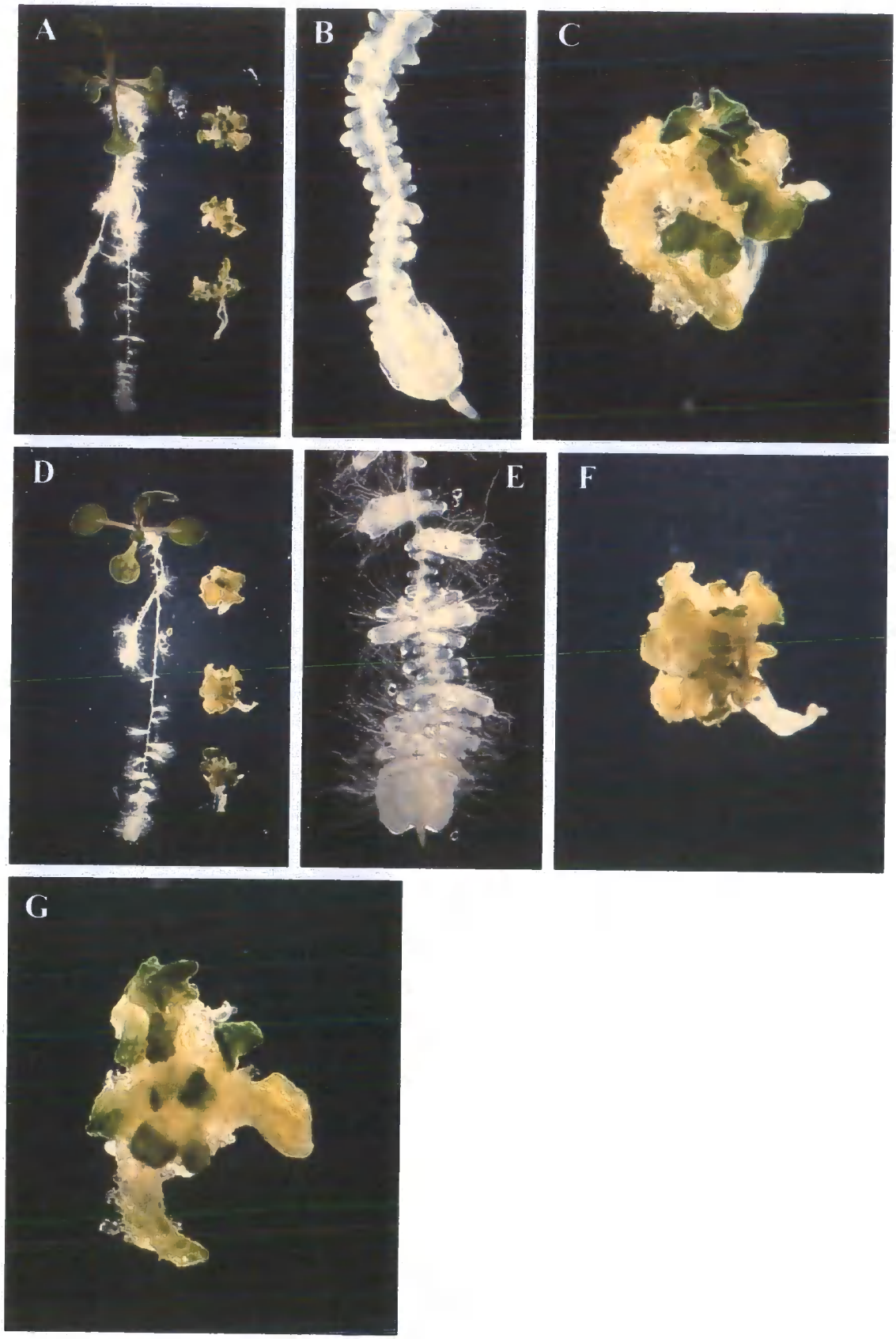
D: (L-R) WS and 3 *hydra2* seedlings.

E: WS (mag. x 8).

F: *hydra2* (mag. x 6).

G: *hydra2* (mag. x 8).

Figure 6.1.2



The *hydra* mutants also showed a callusing response on this auxin, at both concentrations of 2,4-D within 3-5 days of transfer (Fig. 6.1.2, C, F and G). Wild type seedlings only showed evidence of callusing on concentrations of 10μM 2,4-D and above (data not shown).

Together these results indicate that both the *hydra* mutants are hyperresponsive to exogenously applied membrane permeable and non-membrane permeable synthetic auxin analogues. This may result from an increased level of auxin responsiveness or an increased amount of auxin entering the cells, or both.

6.2 The Synthetic Auxin Reporter *DR5* is Abnormally Expressed in the Main Root of *hydra2*

In order to investigate auxin responses *in vivo*, a synthetic auxin reporter gene construct was used to visualize auxin levels within *hydra2* seedlings. The *DR5* construct consists of 7 tandem repeats of an auxin-responsive element fused to the β-glucuronidase (GUS) reporter gene (Ulmasov *et al.*, 1997). The *DR5* reporter is activated rapidly by auxins within the 10^{-8} - 10^{-4} M range, thereby showing where high levels of auxin accumulate within specific tissues or where cells are most sensitive to auxins. Expression of this reporter shows a "maximum" in the root meristem region, in the columella initials of wild-type seedlings (Sabatini *et al.*, 2000, Figure 6.2, A and B).

hydraDR5 seedlings were germinated grown on half-strength MS medium and GUS activity in homozygous *hydra* mutants containing the gene fusion was localised by histochemistry every three days, up to 21 days post-germination.

DR5 expression was abnormal in a developmentally regulated way, with the most dramatic changes seen in the *hydra2* root. In *hydra1* the expression of *DR5* was similar to wild type in the root throughout development (data not shown). *hydra2* showed expression similar to wild type until 12 days post germination. However by day 12, expression started to diminish in intensity and to shift distally in the root tip (Fig 6.2, G to I). This reduction in expression continued to day 15 (Fig 6.2, J and K), and by day 18 there was little or no expression detectable in the primary root tip (Fig 6.2, L-P).

Figure 6.2

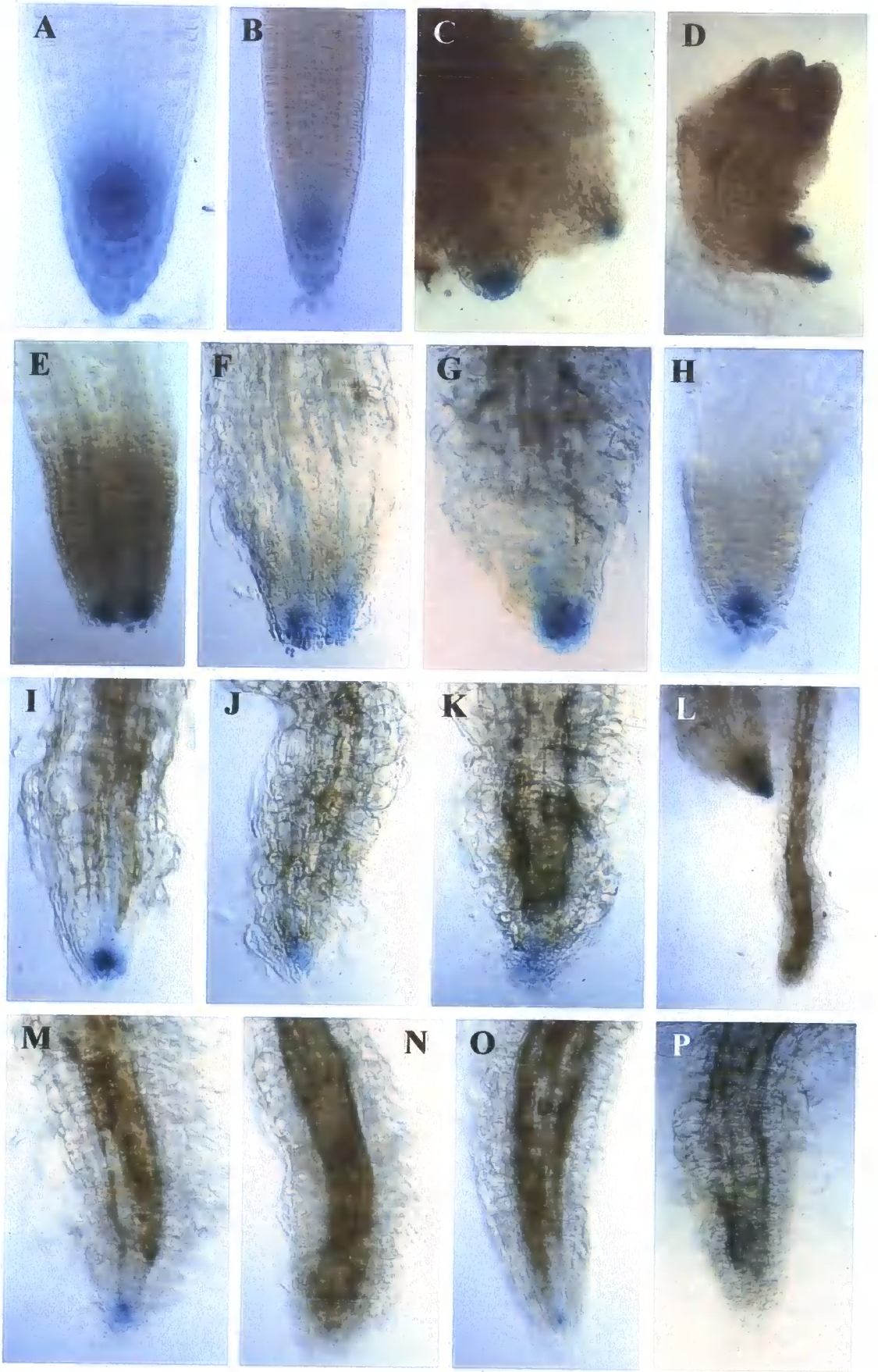
***DR5* Expression in the *hydra2* Root**

A and B: *DR5* expression in wild type seedlings (A mag. x 30, B x 20).

C to P: *hydra2*

- C and D: 3 days after germination (C and D mag. x 12).
- E: 6 days after germination (mag. x 20).
- F: 9 days after germination (mag. x 20).
- G to I: 12 days after germination (G and I mag. x 20).
- J and K: 15 days after germination (J and K mag. x 20).
- L – P: 18 days after germination (L – P mag. x 20).

Figure 6.2



The loss of *DR5* expression shows that the level of auxin or the level of sensitivity to auxin within the *hydra2* root reduces over the same time-frame as the other events seen in the meristem, such as a loss of cell division activity.

6.3 *DR5* Expression in the Main Root of *hydra2* is Rescued by Silver Ions

Silver ions have been shown previously to rescue cell division within the *hydra2* primary root meristem (section 5.2). In order to determine if the inhibition of ethylene signaling could rescue *DR5* expression in the *hydra2* root, *hydra2DR5* seedlings were transferred 6 days after germination onto medium containing either 10μM $AgNO_3$ or 10μM ACC.

When grown in the presence of 10μM $AgNO_3$, *DR5* expression similar to wild type was maintained in the primary root 18 days after germination (see Figure 6.3, D, E and F). Applications of 10μM ACC failed to rescue wild type *DR5* activity in the primary root 18 days after germination (Fig. 6.3, A, B and C), which is consistent with the previous findings that exogenous ACC did not rescue cell division or root growth.

These results suggest that inhibition of defective ethylene signaling rescues defective auxin distribution or signalling in the *hydra2* meristem.

Figure 6.3

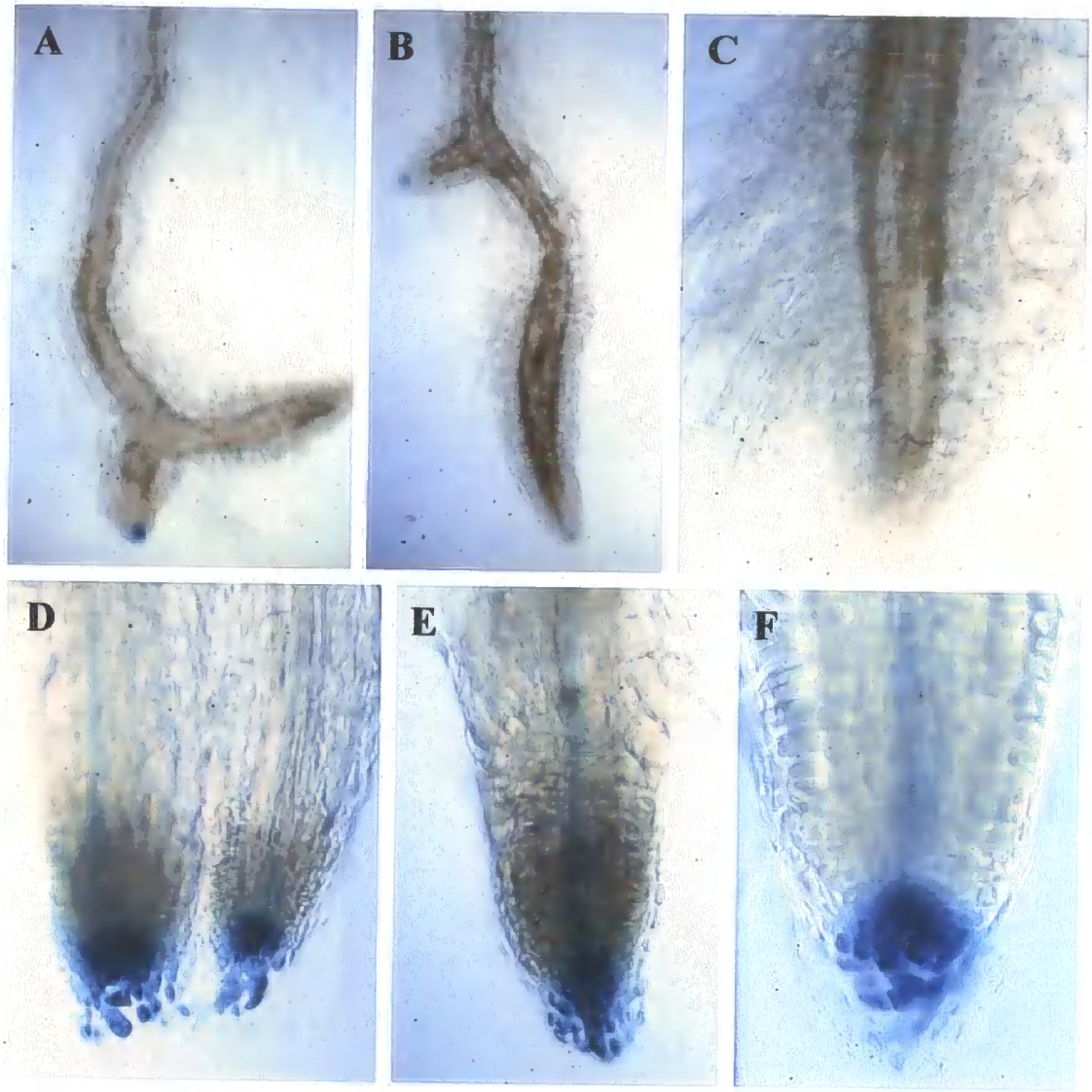
Rescue of *DR5* Expression in the *hydra2* Root by Silver Ions

Seedlings were transferred to either 10μM ACC or 10μM $AgNO_3$ 100nM supplemented medium 6 days after germination, and grown for a further 12 days.

A, B and C: *DR5* expression in *hydra2* seedlings grown on 10μM ACC (A and B mag. x 15, C x 20).

D, E and F: *DR5* expression in *hydra2* seedlings grown on 10μM $AgNO_3$ (D , E and F mag. x 20).

Figure 6.3



6.4 Responses of the *DR5* Reporter in the *hydra* Background to Exogenously Applied Auxins

To further investigate the response of the mutants to exogenous auxins, wild type seedlings and *hydra* mutants containing the *DR5* marker were subjected to different exogenous auxin treatments.

Seedlings were germinated on half-strength MS medium and then transferred to auxin-supplemented medium on ca. 6 days after germination. Wild type seedlings containing *DR5* were grown on 1μM 2,4-D for three days. As seen in Figure 6.4, there was an increase in the number of lateral roots, some of which emerged from the hypocotyl. GUS expression was up-regulated, but was restricted to the tips of the extra lateral roots (Figure 6.4, A and B), with no increase in activity in the shoot region.

Three days after being transferred to medium containing 1μM 2,4-D, the *hydra2DR5* seedlings underwent a callusing response, and showed a massive up-regulation of *DR5* expression throughout the root and shoot regions of the plant/callus (Fig. 6.4, C, D, E and F).

These observations suggest that either more exogenous auxin enters the *hydra2* mutants compared to wild type, or that the mutants are more responsive to exogenous auxin.

Figure 6.4

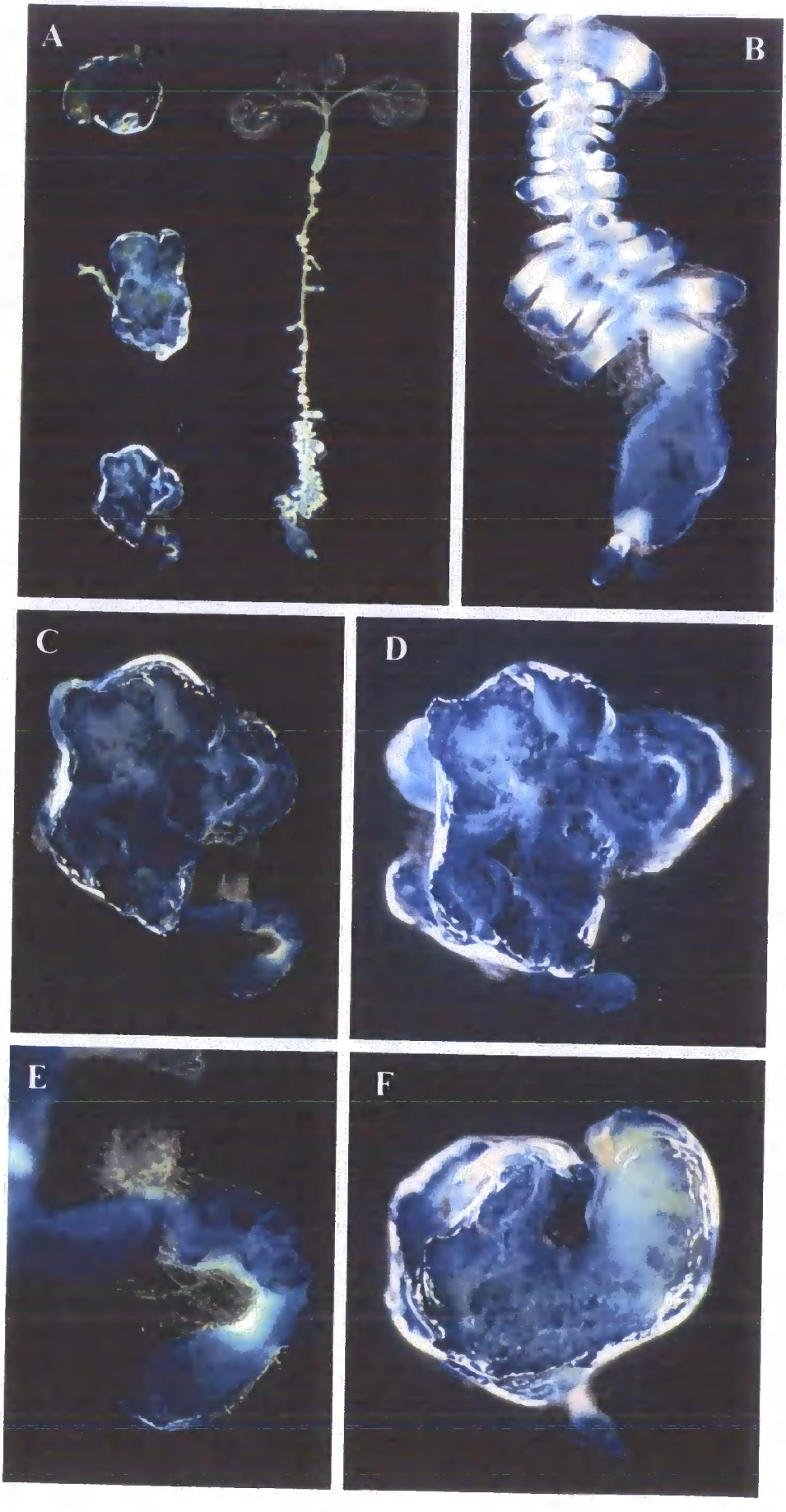


Figure 6.4

Responses of the *DR5* Reporter in the *hydras* to 1μM 2,4-D

Seedlings were transferred to 1μM 2,4-D supplemented medium 6 days after germination, and grown for a further 6 days.

A: (L-R) *DR5* expression in *hydra2* seedlings and a wild type seedling.

B: *DR5* expression in a wild type root (mag. x 10).

C to F: *DR5* expression in *hydra2* undergoing a callusing response (C, D and F mag. x 8, E x 15)

6.5 The *IAA2::GUS* Reporter Gene Shows Abnormal Expression in the *hydra2* Main Root That can be Rescued by Silver Ions

The observations of *DR5* expression in *hydra2* seedlings indicated that there were abnormalities in auxin response within this mutant. To further investigate this, a second auxin response GUS reporter was crossed into the *hydra2* background. *IAA2::GUS* is a reporter construct consisting of the early auxin response *aux/IAA* gene, *IAA2*, linked to GUS (courtesy of Alan Marchant, University of Nottingham), that acts as a marker for early auxin-induced gene expression within the plant (Swarup *et al.*, 2001).

Seedlings were germinated and grown vertically on the surface of half-strength MS medium. GUS activity was analysed by histochemistry every three days. In wild type seedlings, *IAA2::GUS* expression was seen within the vasculature of the main root, developing lateral roots and in the root tip itself (Figure 6.5, A – D). In *hydra2*, expression was similar to wild type from 3 days to 15 days post germination (Fig. 6.5, E-N), with intense GUS activity seen in the meristem itself. From day 18 however, GUS activity in the main root meristem declined (Fig. 6.5, O, P, Q, R, S and T), such that there was no staining at all in the main root by day 24 (Fig. 6.5, U, V and X).

The reduction of auxin responsiveness of the roots, seen in the *DR5* and *IAA2* experiments, occurs within the same time frame, and parallels the loss of cell division activity within the meristem associated with defective ethylene signalling.

In order to determine if inhibiting ethylene signalling could rescue wild type *IAA2* expression, *hydra2IAA2::GUS* seedlings were transferred from half-strength MS medium 6 days after germination onto medium containing 10μM $AgNO_3$. Wild type *IAA2::GUS* expression was seen in the primary root of *hydra2* seedlings 21 days after germination (Figure 6.5, Y and Z). This rescue of auxin responsiveness further supports the view that the *hydra2* root meristem is defective in ethylene responses, resulting in a loss of auxin signalling control.

Figure 6.5

hydra2IAA2::GUS

A to D: *IAA2::GUS* expression in wild type seedlings at various ages, the expression pattern is consistent throughout development (A and C mag. x 15, B x 40, D x 30).

E to P: *IAA2::GUS* expression in *hydra2* seedlings (E, F, G, H, I, J, L, M, N and P mag. x 20, K and O x 15).

E and F: 3 days after germination

G and H: 6 days after germination

I and J: 9 days after germination

K and L: 12 days after germination

M and N: 15 days after germination

O and P: 18 days after germination

Figure 6.5.1

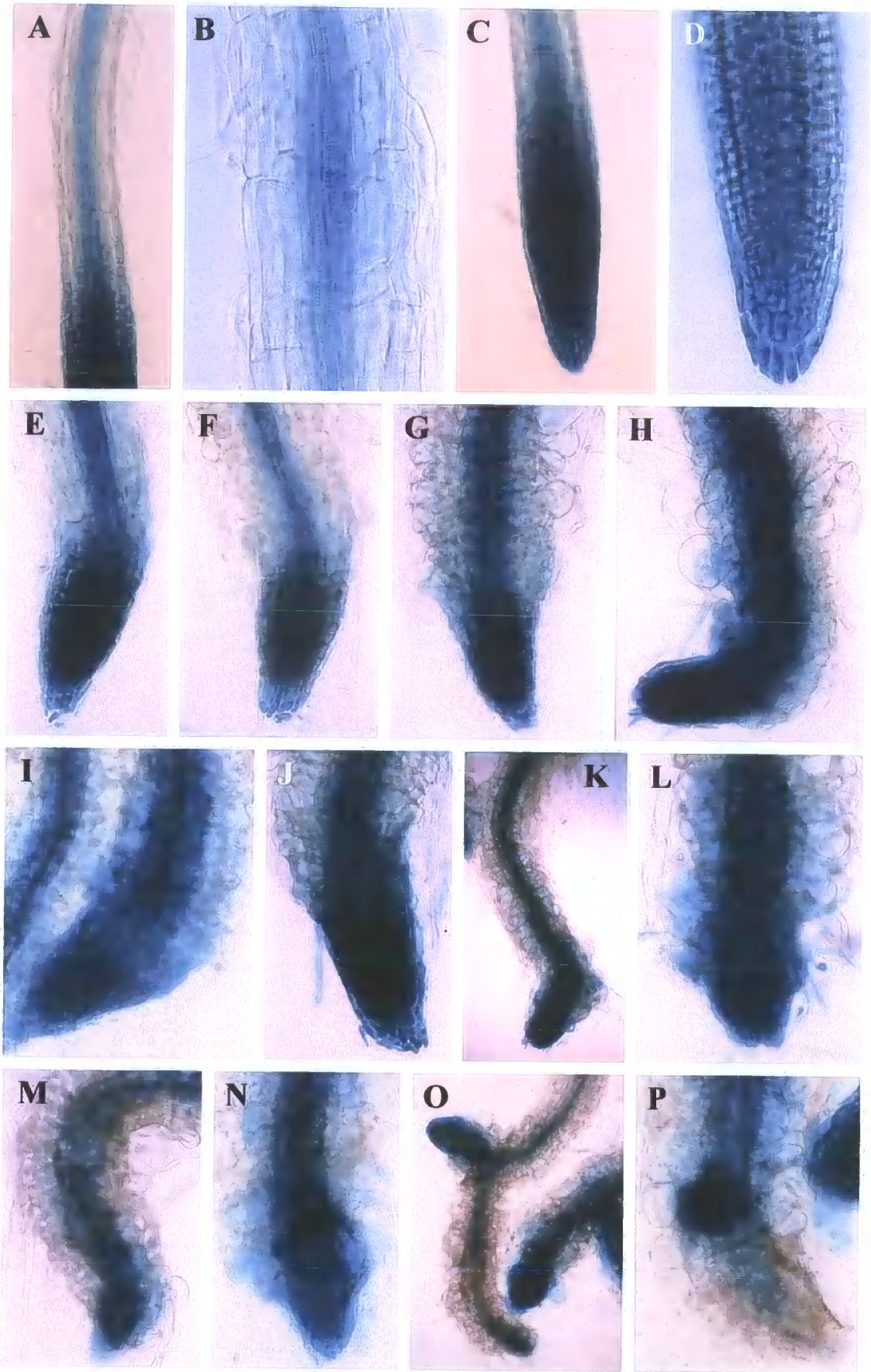


Figure 6.5

hydra2IAA2::GUS

Q to X: *IAA2::GUS* expression in *hydra2* seedlings (Q, R, S and T mag. x 20, U, V and X x 15).

Q: 18 days after germination

R, S and T: 21 days after germination

U, V and X: 24 days after germination

Y and Z: *IAA2::GUS* expression in *hydra2* seedlings grown on 10μM $AgNO_3$, 21 days after germination (mag. x 20).

Figure 6.5.1

6.6 Mutations in the *AXR1* Gene Cause Auxin Resistance Which Rescues the *hydra* Mutant Phenotype

In order to investigate the influence of abnormal auxin responsiveness *in vivo*, a mutation in *AXR1* (*axr1-12*), a gene required for correct auxin responses (Lincoln *et al.*, 1990; Rouse *et al.*, 1998), was introduced into both *hydra1* and *hydra2* backgrounds.

Double mutants were isolated from F2 populations that contained *axr1-12* mutants, identified as seedlings phenotypically similar to wild type but which were insensitive to inhibition of root growth by auxin (0.1μM 2,4-D). *hydra*-like seedlings were also identified for further analysis.

hydra1

30 *hydra*-like seedlings were grown on vertical plates under standard growth conditions, and the length of the primary root was measured every three days so as to determine the segregation ratios of the population. 7 out of 30 (approx. 1/4) seedlings had a longer root (18 DAG, 25.71 ± 1.97), than *hydra1* mutants (18 DAG, 21.0 ± 0.89). This population corresponded to the predicted double homozygous *hydra1axr1-12* double mutants, indicating that the effect of *axr1-12* on *hydra1* root growth is only seen when mutants are homozygous for the *axr1-12* mutation. The increase in root growth seen in the putative double mutants was 20-30% greater when compared to *hydra1* root growth (see Figure 6.6.1).

Figure 6.6.1 *hydra1axr1-12* Root Growth

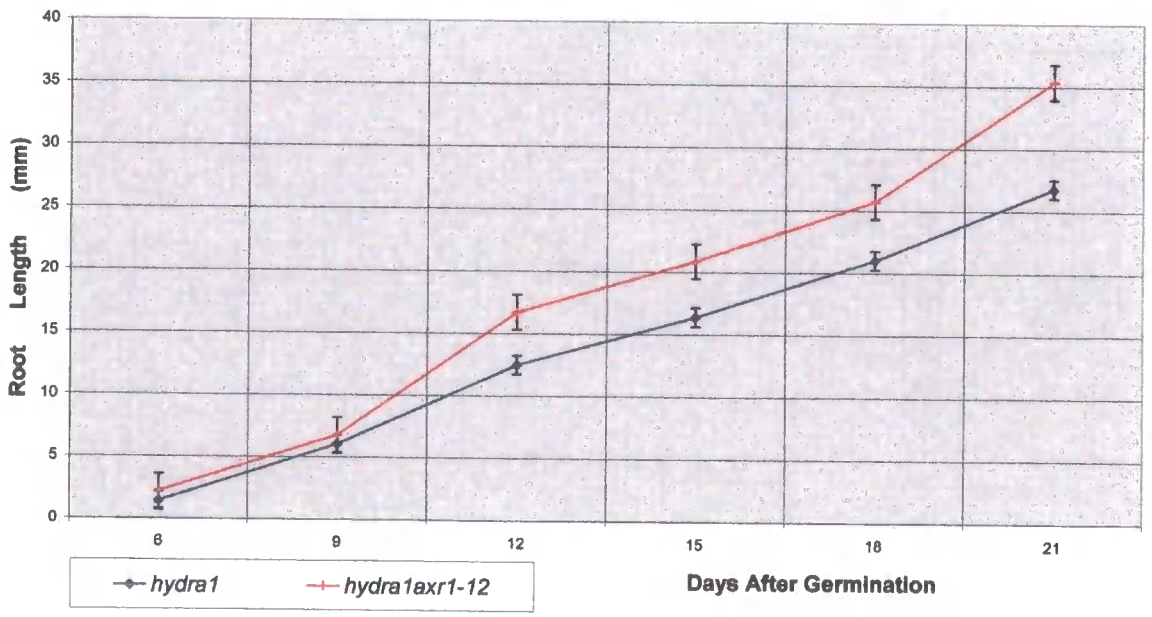


Figure 6.6.2

***hydra1axr1-12* Double Mutants**

All seedlings shown are 18 days after germination.

A: (L-R) predicted *hydra1axr1-12* double mutant and a *hydra1* seedling.

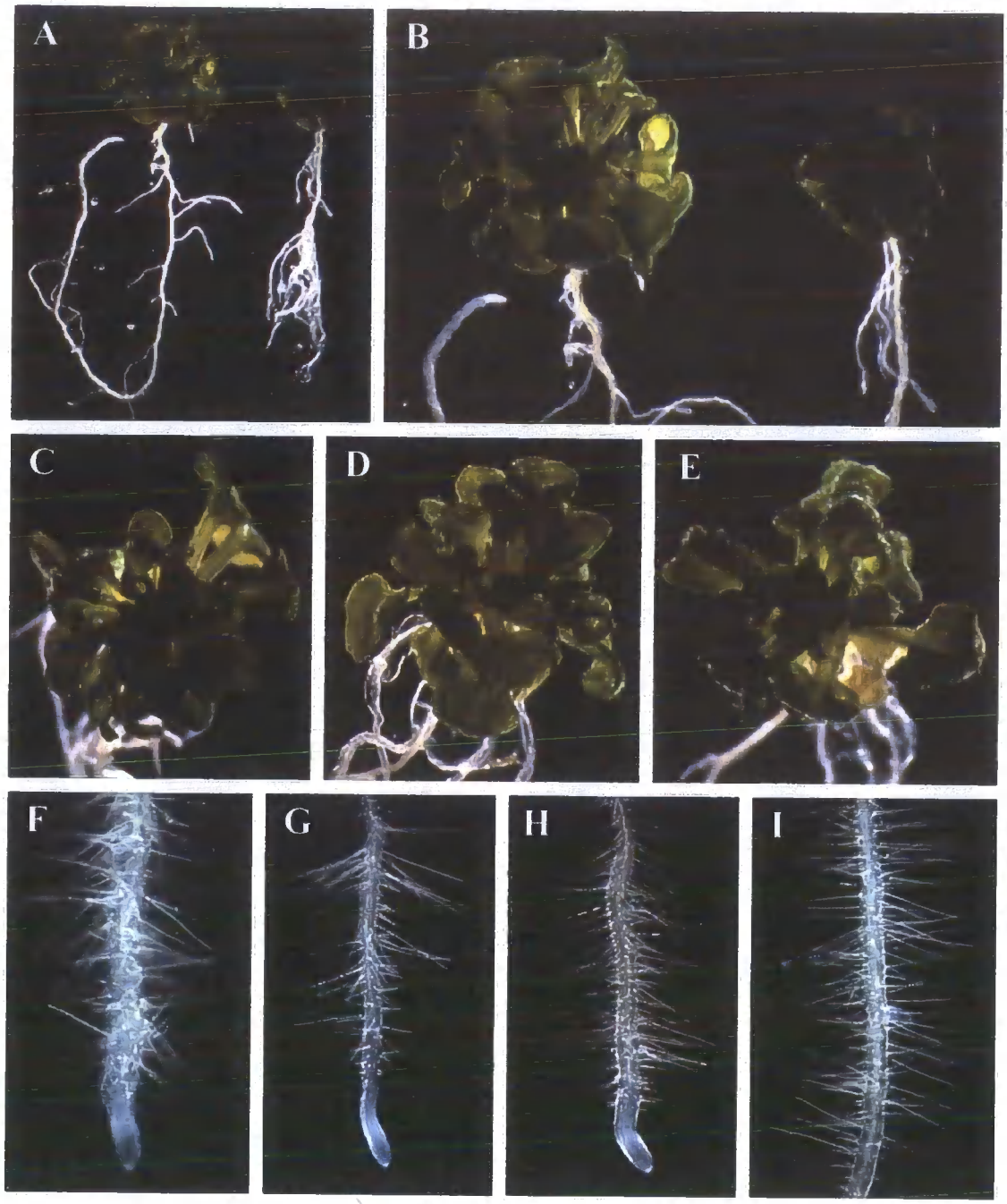
B: (L-R) predicted *hydra1axr1-12* double mutant and a *hydra1* seedling.

C, D and E: Predicted *hydra1axr1-12* double mutants.

F: Root hair patterning in *hydra1* (mag. x 15).

G, H and I: Root hair patterning in predicted *hydra1axr1-12* double mutants (mag. x 15).

Figure 6.6.2



Root hair patterning in the putative double mutants showed a recovery to wild type patterning (Fig. 6.6.2, F, G, H and I). This indicates that auxin or ethylene may be involved in generating the root hair phenotype in the *hydra* mutants, as *axr1-12* mutants are also partially resistant to ethylene (Lincoln *et al.*, 1990).

Putative double mutants were identified on the basis of root phenotype. The shoot was also studied to determine if the *hydra* phenotype was modified by the *axr1-12* mutation. 23 out of the 30 *hydra*-like seedlings (approx. 3/4) originally examined for the root phenotype showed some shoot recovery, with longer petioles and more expanded leaves. This suggests that the effect of *axr1-12* on *hydra1* is semi-dominant. This is in contrast with the segregation of the root phenotype, which showed that only double homozygous *axr1-12 hydra1* seedlings had longer roots. Putative homozygous *axr1-12 hydra1* double mutants not only had the longest roots, but also showed the greatest recovery in shoot development. As seen in Figure 6.6.2, petiole elongation created a more expanded rosette (Fig. 6.6.2, B, C, D and E). Microscopic examination of the vascular patterning of the leaves showed it was more organised and wild type in appearance (data not shown, observations made by Margaret Pullen, University of Durham; Souter *et al.*, 2002).

hydra2

In an F2 population of 57 seedlings, 45 (approx. 3/4) had longer roots than *hydra2* seedlings. This represented two distinct populations of seedlings based on primary root length, two-thirds accounting for the putative *hydra2/AXR1/axr1* heterozygous seedlings, with the other third the putative *hydra2axr1/axr1* homozygous population. When the root growth of these two populations was compared with *hydra2* (21 DAG, 4.29 ± 0.18), it was clear that the putative double homozygous mutants had longer roots (21 DAG, 10.3 ± 0.44), and the putative heterozygous *axr1-12* seedlings, an intermediate phenotype (21 DAG, 7.04 ± 0.32) (Figure 6.6.3).

Shoot growth in the *hydra2axr1-12* double mutants was not rescued to the extent seen for *hydra1*. Leaf vascular patterning was however rescued (data not shown, observations made by Margaret Pullen, University of Durham; Souter *et al.*, 2002).

Figure 6.6.4

***hydra2axr1-12* Double Mutants**

A: (L-R) 2 *hydra2* mutants and 3 predicted *hydra2axr1-12* double mutants, 18 days after germination.

B: (L-R) *hydra2*, predicted *hydra2axr1-12* heterozygote, and predicted *hydra2axr1-12* double homozygote, 18 days after germination.

C and D: Root hair development in predicted *hydra2axr1-12* double homozygous mutants, 18 days after germination (mag. x 15).

Figure 6.6.4

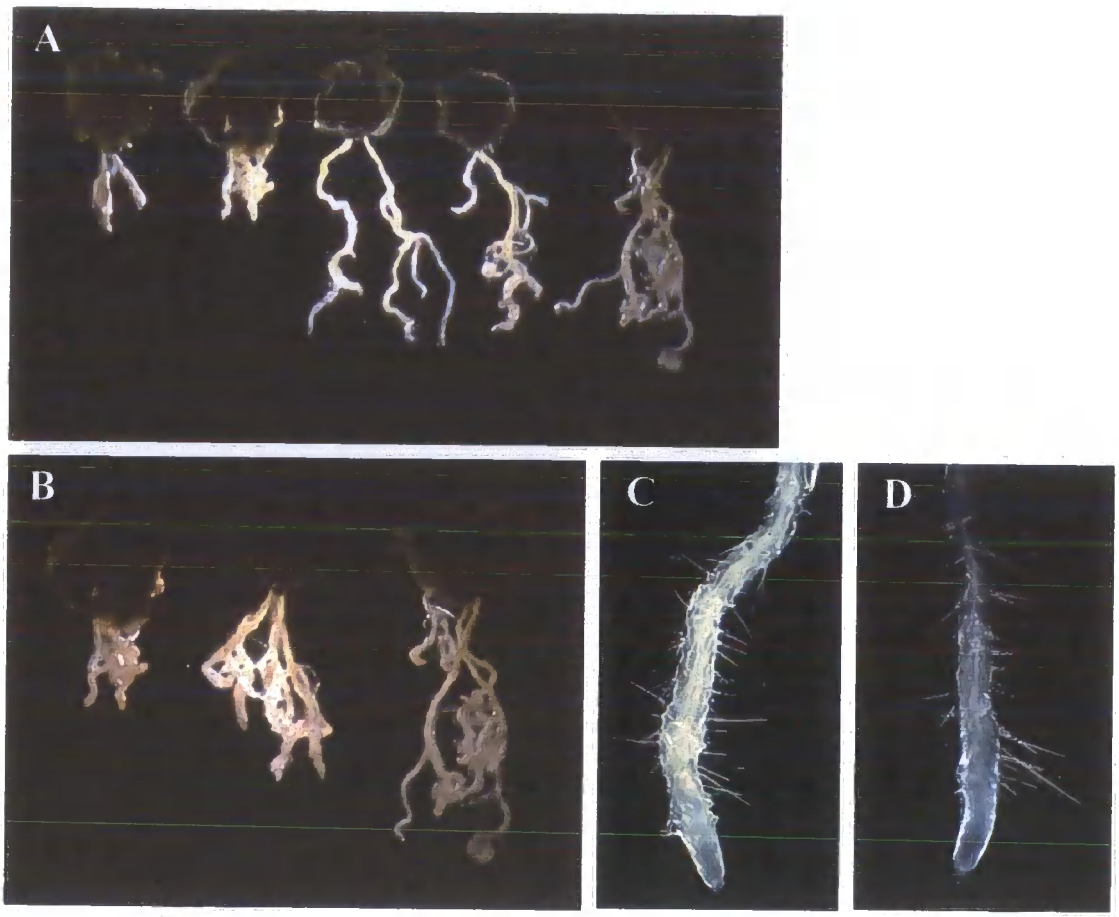
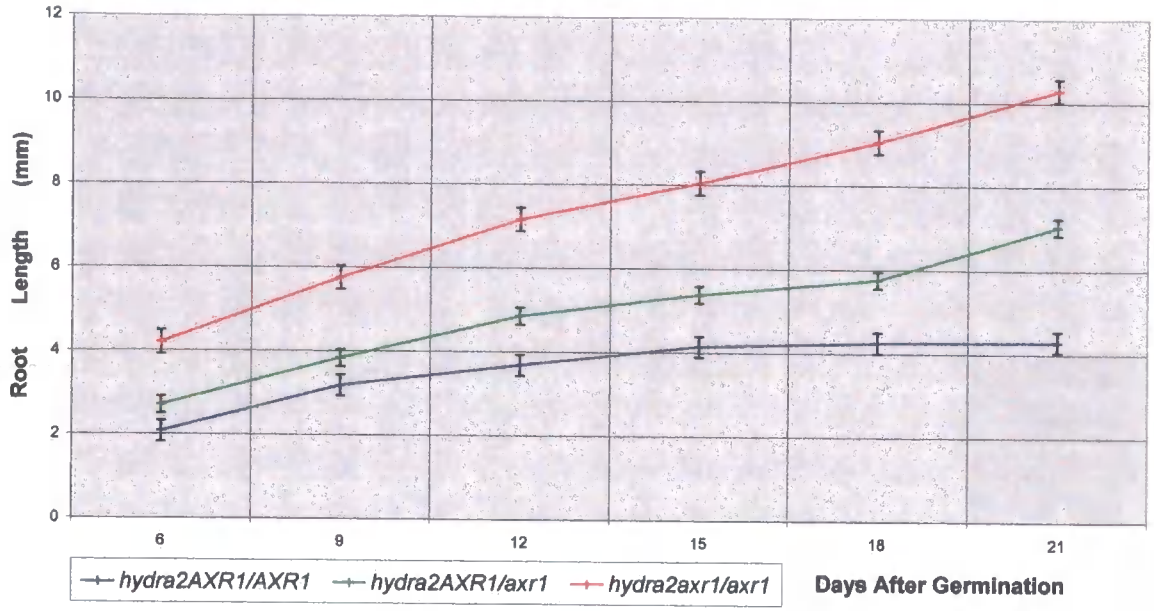


Figure 6.6.3 *hydra2axr1-12* Root Growth



Root growth was clearly rescued in the *hydra2axr1-12* mutants (Figure 6.6.4, A and B), and the patterning of roots hairs was more wild type, such that there were fewer hairs initiating, and those that did initiated further back from the root tip than in *hydra2*. There were no branched root hairs detectable (Fig. 6.6.4, C and D).

These results show that reducing *AXR1* gene function partially rescued the root and shoot phenotype of the *hydra* mutants.

6.7 Mutations in *AXR1*, but not in *EIN2* Stop the Callusing Response of the *hydras* to Exogenous Auxin

When *hydra* mutants were transferred onto auxin-supplemented medium, they showed a callusing response within 3-5 days (section 6.1). In order to determine a role for the *AXR1* and *EIN2* gene products in this response, *hydra axr1-12* and *hydra ein2* double homozygous mutants were transferred ca. six days after germination onto medium containing 1 μM 2,4-D and the callusing response was investigated.

It was found that *hydra2axr1-12* double mutants had failed to undergo the callusing response by six days incubation on 1 μM 2,4-D (Figure 6.7, A, B and C). Both

Figure 6.7

***axr1-12* and *ein2* Double Mutants Grown on Exogenous Auxin**

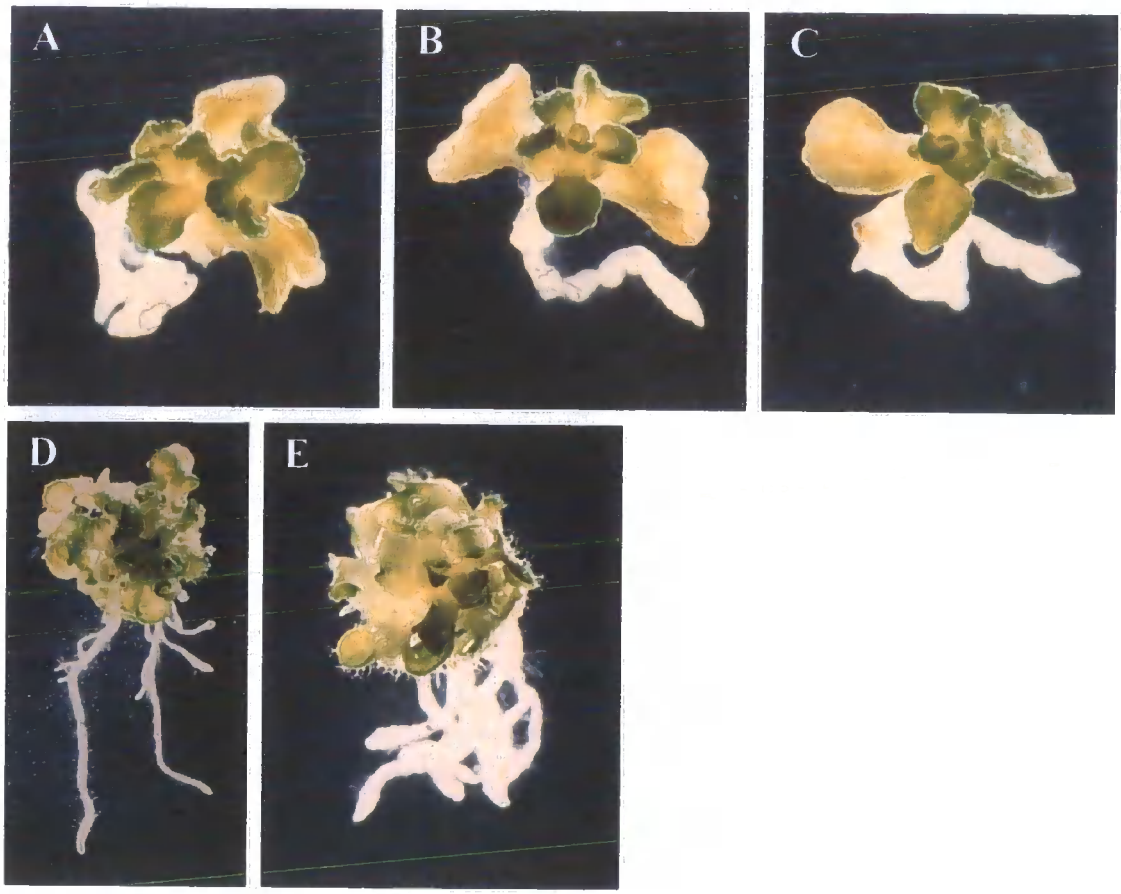
Seedlings were transferred to 1μM 2,4-D supplemented medium 6 days after germination.

A, B and C: Predicted *hydra2axr1-12* double mutants, 6 days after transfer (mag. x 6).

D: Predicted *hydra1ein2* double mutant, 9 days after transfer (mag. x 5).

E: Predicted *hydra2ein2* double mutant, 9 days after transfer (mag. x 5).

Figure 6.7



hydra1ein2 and *hydra2ein2* double mutants showed a delayed callus response, taking 9 days before the response could be detected (Fig. 6.7, D and E).

These observations show that the callusing response requires the activity of both *AXR1* and *EIN2* genes.

6.8 A Mutation in the *AXR3* Gene Suppresses Root Hair Development in *hydra2*

In order to investigate further the defects in auxin signalling, an *AUX/IAA* mutant was crossed into the *hydra2* background. The *auxin resistant3-1* (*axr3-1*) mutant is a semi-dominant recessive mutation in the *IAA17/AXR3* gene, encoding an AUX/IAA protein (Ouellet *et al.*, 2001). The *axr3-1* allele is a mutation in Domain II (responsible for the degradation of this negative regulator of auxin induced gene transcription) and results in a 7-fold higher stability in the protein, as it cannot be degraded by the ubiquitin-proteasome machinery (Ouellet *et al.*, 2001). The wild type IAA17/AXR3 protein is expressed in the elongation zone of the root tip, and is degraded before root hairs can start to develop in the differentiation zone. *AXR3* is therefore a negative regulator of root hair development so that in the semi-dominant *axr3-1* allele, no root hairs are seen. The mutant is also agravitropic.

Double mutants were identified in F2 populations that contained *axr3-1 mutants*, seedlings with hairless and agravitropic roots. Putative *hydra2axr3-1* double mutants were identified as phenotypically modified *hydra*-like seedlings.

There was no obvious effect of the *axr3-1* mutation upon the shoot development of *hydra2*, however there was a considerable alteration in the root phenotype. *hydra2axr3-1* double mutants had hairless agravitropic roots. They could not be accurately measured for root length as the roots curled up due to their agravitropic nature, making non-invasive root measurements on the same plants impossible. However they continued to grow beyond the length of *hydra2* mutants, suggesting that cell division activity within the meristem had been rescued. The roots showed a complete lack of root hairs, and the root epidermis also showed a rescue of the swollen epidermal bubbling effect seen in *hydra2* mutants (Figure 6.8, A to D).

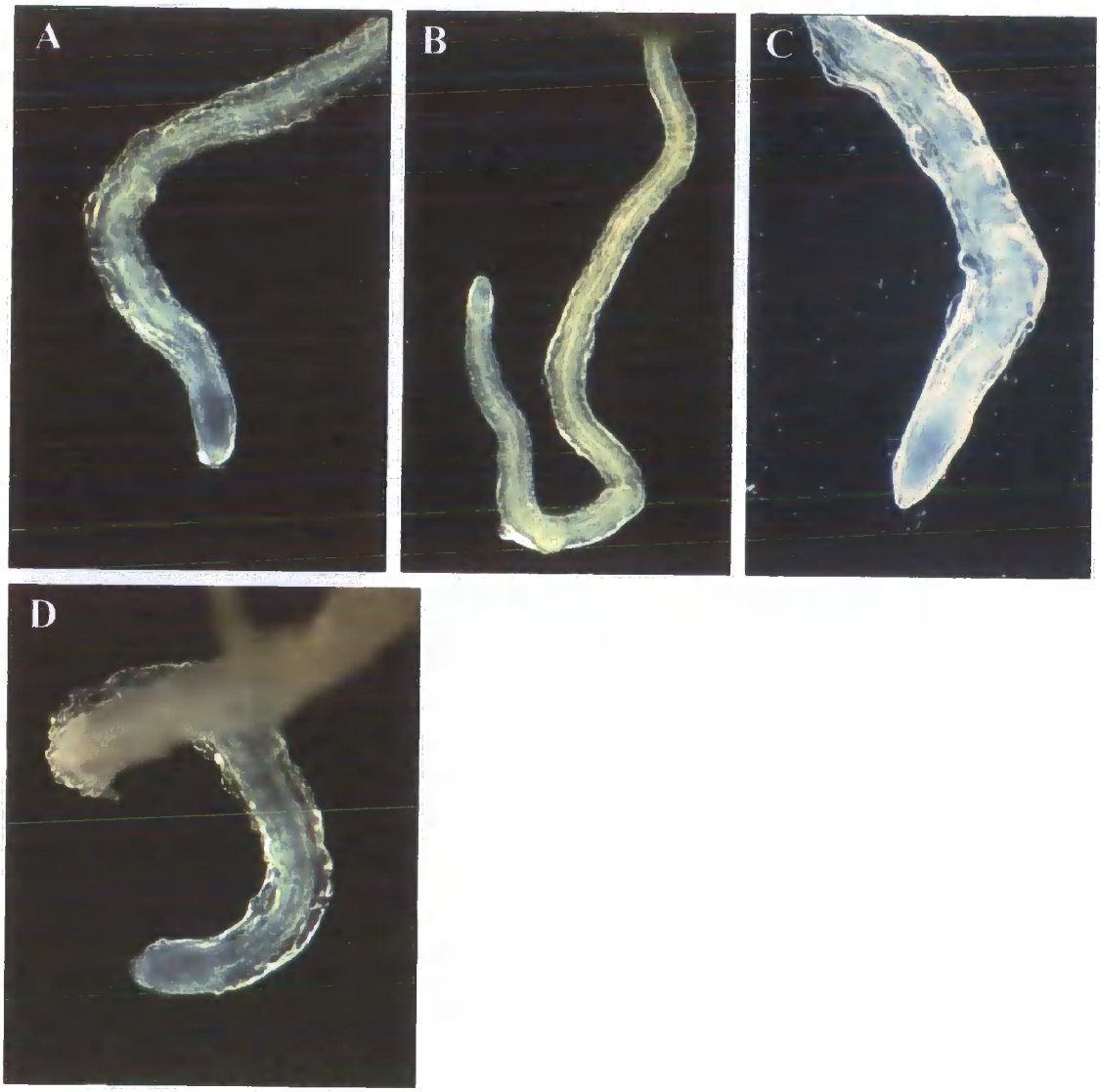
Figure 6.8

***hydra2axr3-1* Double Mutants**

A: *axr3-1* mutant root totally lacking root hairs, 12 DAG (mag. x 15).

B - D: Predicted *hydra2axr3-1* double mutants, 12 DAG (mag. x15).

Figure 6.8



The root hair phenotype of the *hydra2axr3-1* mutants indicated that the root hair phenotype of *hydra2* could be due to defects in both ethylene and auxin responses, whilst the rescue of the root meristem activity confirmed observations of the *axr1-12* mutants, namely that aberrant auxin responsiveness may contribute to the loss of cell division activity in the *hydra* meristem.

6.9 Pharmacological Inhibition of the Influx Carrier does not Alter the Ectopic Expression of the DR5 Reporter in Response to Exogenously Applied Auxin

The observed callusing response and enhanced GUS activity seen when *hydraDR5* mutants were grown in the presence of auxins suggested that they were either more sensitive to exogenous auxins, or they took up more auxin from the growth medium. The experiments described above showed that the *hydra* phenotype could be suppressed by reducing auxin response, and so to investigate the possibility of an enhanced uptake of exogenous auxin, 3 day-old wild type, *hydra, hydra2DR5* and wild type *DR5* seedlings were transferred to plates containing 10μM 1-naphthoxyacetic acid (1-NOA), a specific inhibitor of the auxin influx carrier *AUX1* (Parry *et al.*, 2001). The plants were allowed to grow for three days on half-strength MS medium in the presence of the inhibitor (10μM), and then transferred to plates containing 10μM 1-NOA plus 1μM 2,4-D, a synthetic auxin analogue that requires the influx carrier to enter the cell efficiently (Delbarre *et al.*, 1996). Seedlings were grown for a further three days, when plants were stained for GUS activity. Both mutant and wild type seedlings were also grown in parallel on 10μM 1-NOA, so that the staining patterns could be compared.

Figure 6.9 shows that the wild types grown in the presence of 1-NOA and 2,4-D did not produce as many laterals and exhibited reduced *DR5* activity as they did on 2,4-D supplemented media (Figure 6.9, A, B, C and D) (compare with Fig. 6.4). However, both *hydra1* and *hydra2* showed a callusing response (Fig. 6.9, A, B and E), and a massive up-regulation of *DR5* expression throughout the plant in the presence of 1-NOA and 2,4-D (Fig. 6.9, C and H). *DR5* GUS expression in *hydra1* and *hydra2* was up-regulated to the same level as when the plants were grown on 2,4-D alone (Fig. 6.9, G).

Figure 6.9

Expression of *DR5* in Seedlings Treated with 10μM 1-NOA and 1μM 2,4-D

A, B, C, D, E and H show seedlings grown on 10μM 1-NOA for three days (6 DAG) and then transferred to 10μM NOA and 1μM 2,4-D, for a further three days.

A: (L-R) C24 and 3 *hydra1* seedlings.

B: (L-R) WS and 3 *hydra2* seedlings.

C: (L-R) *DR5* expression in 2 *hydra2* seedlings and a WS seedling.

D: *DR5* expression in a wild type root, note the accumulation of GUS staining at the tip of the extra lateral roots, compare with Figure 6.4, B (mag. x 10).

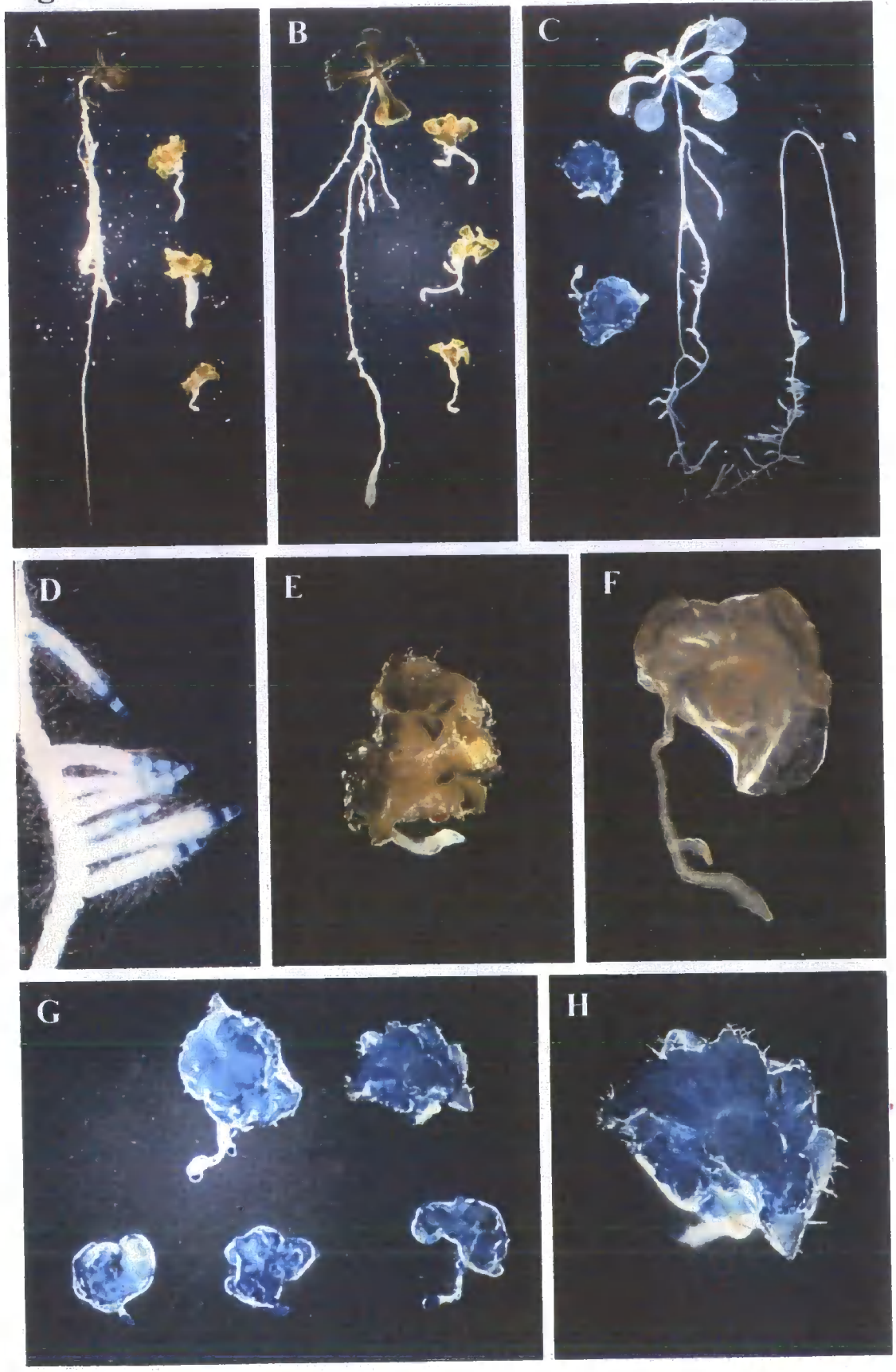
E: *hydra1* seedling (mag. x 6).

F: *DR5* expression in a *hydra2* seedling grown on 10μM 1-NOA only (mag. x 6).

G: *DR5* expression in *hydra2* seedlings (L-R) upper two grown on the combined 1-NOA and 2,4-D treatment plates, whilst the bottom three seedlings have been grown on 2,4-D supplemented media only.

H: *DR5* expression in a *hydra2* seedling (mag. x 6).

Figure 6.9



When *hydra1* and *hydra2* seedlings were grown in the presence of 1-NOA only, there was no up-regulation of *DR5* expression in the shoot or the root as seen in the presence of exogenous 2,4-D, and *DR5* expression was restricted to the root tip only (Fig. 6.9, F). This indicates that the ectopic up-regulation of *DR5* expression was due to the presence of 2,4-D, and not 1-NOA. This suggests either that more 2,4-D was entering the seedlings, or that they have a heightened sensitivity to the low amount of 2,4-D that leaks in. The promotion of lateral root development in the wild type seedlings suggests that some 2,4-D was able to enter the plants, however this may only constitute a very low level. The effect on the *hydras* was somewhat different, suggesting that either 2,4-D is able to bypass the 1-NOA-sensitive auxin influx carrier in the *hydra* mutants to a greater extent than in the wild type, or that the influx carrier had become 1-NOA insensitive within the *hydra* background, or that the *hydra* mutants were more sensitive to the auxin by-passing the *AUX1* carrier.

6.10 *aux1* Mutants are Differentially Rescued in the *hydra* Backgrounds

To further understand the interaction of the influx carrier and the phenotype of the *hydra* mutants, a genetic approach was taken. Double mutants were made with both *aux1-7* and *aux1-100*, two different alleles of *AUX1*, a member of the auxin influx carrier family (Marchant *et al.*, 1999). The *aux1-7* allele responds in growth assays as a null allele, although it is not a complete knock-out and produces a similar level of protein to wild type; however it is a non-functional protein (A. Marchant, University of Nottingham, personal communication). *aux1-100*, however, represents a T-DNA insertion mutant that creates a complete null allele; there is no *AUX1* protein detectable by Western blotting in *aux1-100* mutant seedlings (A. Marchant, University of Nottingham, personal communication).

aux1 mutants are morphologically similar to wild type plants, however they are agravitropic. Approximately 20 wild type-looking seedlings were taken from each of the F2 lines that contained *hydra* mutants. The seedlings were transferred and aligned on fresh square half-strength MS plates to be grown vertically on the surface of the agar. The square plates were turned 90° and left for 24 hours. Agravitropic seedlings were selected by their inability to realign to the gravity vector. All lines that failed to show

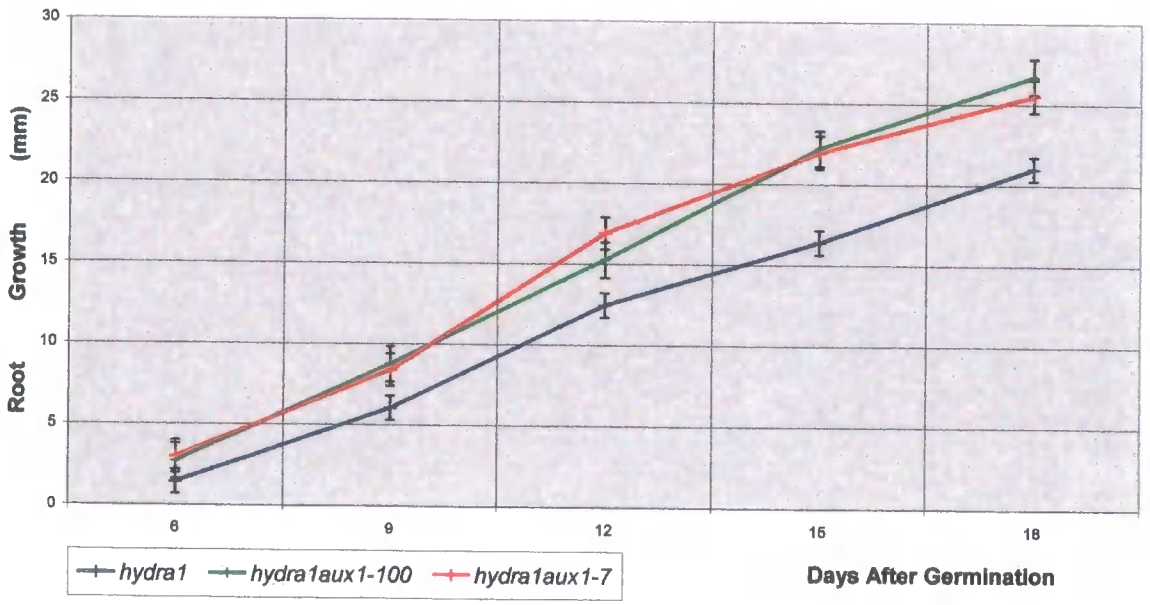
agravitropic responses were discarded, and *hydra aux1* double mutants were selected from the F2 lines that contained agravitropic seedlings. Root growth assays were used to identify segregating populations of modified *hydra* phenotypes, which represented the putative *hydra aux1* double mutants.

hydra1aux1-7 and *hydra1aux1-100*

10 out of 43 putative *hydra1aux1-7* seedlings (approx. 1/4), and 10 out of 39 putative *hydra1aux1-100* seedlings (approx. 1/4) had a longer primary root than *hydra1* seedlings. These segregating populations represent the predicted *hydra1aux1-7* and *hydra1aux1-100* double homozygous mutants, indicating that both the *aux1-7* and *aux1-100* mutations affected *hydra1* root growth only when homozygous for the mutation.

In order to measure the effects of the aux1 mutations on hydra1 root growth, seedlings were grown on vertical half-strength MS plates, and the primary root was measured every three days until 18 days post germination. When the root growth of the putative double mutants was compared with *hydra1* root growth (Figure 6.10.1), it showed that both *aux1* mutations caused an identical increase in root growth.

Figure 6.10.1 Root Growth in *hydra1aux1* Double Mutants



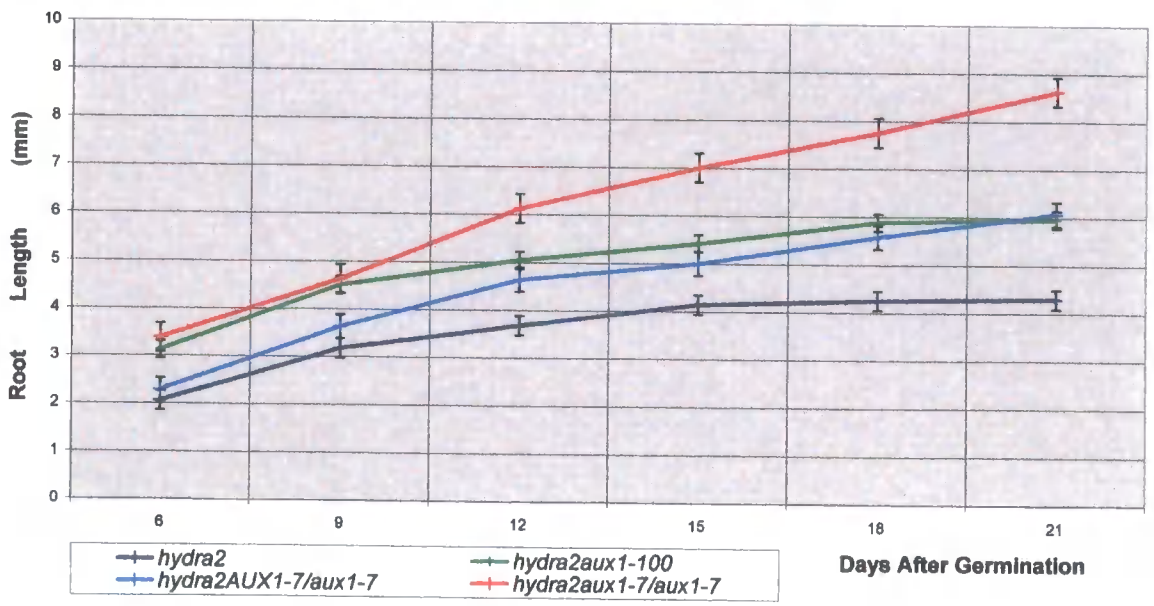
hydra2aux1-7

11 out of 14 putative *hydra*-like seedlings (approximately 3/4) had longer roots than normal *hydra2* mutants. This segregation ratio indicated that the population represented two of the expected three segregating populations, the predicted *hydra2AUX1-7/aux1-7* heterozygous mutants, as well as the predicted *hydra2aux1-7/aux1-7* double homozygous mutants. When this mixed population was analysed for root growth, two different populations were identified based upon expected ratios. 4 out of 11, the predicted *hydra2aux1-7* double homozygotes, possessed a distinguishably longer root than the remaining 7 seedlings, predicted to be heterozygous for the *aux1-7* mutation (Figure 6.10.3 below). The *aux1-7* mutation therefore had a semi-dominant effect upon the *hydra2* root meristem, which is different from the effect seen in *hydra1*, yet is consistent with the effects of the *axr1-12* and *ein2* mutations. This is indicative of the difference in the activity of the root meristems in the respective *hydra* mutants.

hydra2aux1-100

12 out of 40 putative *hydra*-like seedlings (approx. 1/4) had longer roots than *hydra2* mutants, which represented the predicted *hydra2aux1-100* double homozygous mutants. This segregation ratio indicated that the *aux1-100* mutation only had an effect upon *hydra2* growth in the homozygous state, which is different from the effect of the *aux1-7* mutation upon *hydra2* root growth mentioned above. The root growth of both double mutants is shown below in Figure 6.10.2.

Figure 6.10.2 Root Growth in *hydra2aux1* Double Mutants



Gravity Responses

Because *aux1* mutants are agravitropic, the mutants were subjected to a gravity response assay after the segregation analysis had been performed. Roots of six day old seedlings were aligned using a dissecting microscope upon the surface of half-strength MS medium, ensuring that all root tips were pointing to the bottom of the plate. The plate was then turned 180° (point of gravistimulation) and then checked every 30 minutes for evidence of a response.

Wild type seedlings responded to the gravity stimulus, and a clear re-orientation of growth towards the gravity vector was seen within 30 minutes (data not shown). *aux1-7* and *aux1-100* mutants failed to respond to the stimulus, and showed no consistent direction of growth (data not shown). Putative *hydra1aux1-7* double mutants responded and started to reorient the root tip within 60 minutes of gravistimulation, eventually pointing towards the gravity vector. Putative *hydra1aux1-100* double mutants also responded to the gravity stimulus, although they took 90 minutes to initiate a re-orientation, and they went beyond the gravity vector before realigning and then passing it again. This 'over-steering' response continued throughout the assay.

Putative *hydra2aux1-7* double mutants responded and re-oriented their root tips within 90 minutes of gravistimulation and grew directly towards the gravity stimulus (data not shown). This delayed response reflects the difference in root growth between the putative *hydra1aux1-7* and putative *hydra2aux1-7* double mutants. Putative *hydra2aux1-100* double mutants reoriented their root tips within 120 to 150 minutes of gravistimulation (data not shown), which was considerably later than putative *hydra1aux1-100* and putative *hydra2aux1-7* double mutants. Once re-oriented completely they grew slowly towards the stimulus, and this may also be attributable to the reduced root growth in this double mutant when compared with the others.

These findings represent a rescue, although varying in degree, of the agravitropic phenotype of the *aux1* mutants.

6.11 The Auxin Efflux Carrier Proteins PIN1 and PIN2 Show Correct Tissue and Cellular Localization in the Main Root of the *hydra* Mutants

The auxin influx carrier allows auxin into the cell at a faster rate than it can diffuse across the membrane. The efflux carrier however, gives direction to auxin transport by being preferentially localised within the cell, so that auxin is preferentially transported out of the cell in that particular direction. Consequently the cumulative effect of the localisation of the efflux carrier is a stream of auxin moving through the tissue (Muller *et al.*, 1998). Localisation of the efflux carrier can therefore show the direction auxin is travelling in through the plant.

Following from the observations made above of the abnormal interactions with the auxin influx carrier, it was important to establish if there was any abnormal localisation of the PIN proteins in the *hydra* mutants.

The PIN proteins have their own specific cell localisations, and are delivered by precise vesicle transport to the plasma membrane (Geldner *et al.*, 2001). The tissue-specific expression of the PIN proteins also allows them to be used as cell identity markers as well indicators of normal correctly directed vesicle transport. Experiments were performed to determine whether members of the *PIN* gene family were expressed within the correct tissues as well as the proteins being localised correctly within the specific cell type.

In order to carry out this work, a collaboration was initiated with Dr Klaus Palme's research group at the Max-Planck-Institut für Züchtungsforchung, Köln, Germany, which raised the *PIN*-specific antibodies. Seeds were taken to their laboratories and germinated under standard conditions consistent with those used in Durham. All immunolocalisation experiments were performed in collaboration with Jiri Friml.

Seedling roots of different ages were stained with antibodies raised to *PIN1* and *PIN2*. In the wild type *Arabidopsis* root, *PIN1* localises to the basal (distal) membrane of vascular cells (Galweiler *et al.*, 1998), whilst *PIN2* localises to the apical (proximal) membrane in the root cortex, epidermis, and lateral root cap (J. Friml, personal communication), as well as the outside lateral wall of the cortex (Muller *et al.*, 1998).

Figure 6.11.1

PIN1 Immunolocalisation

A, B and C: Localisation of PIN1 in C24.

D, E and F: Localisation of PIN1 in *hydra2*.

G: Localisation of PIN1 in *hydra1*.

Figure 6.11.1

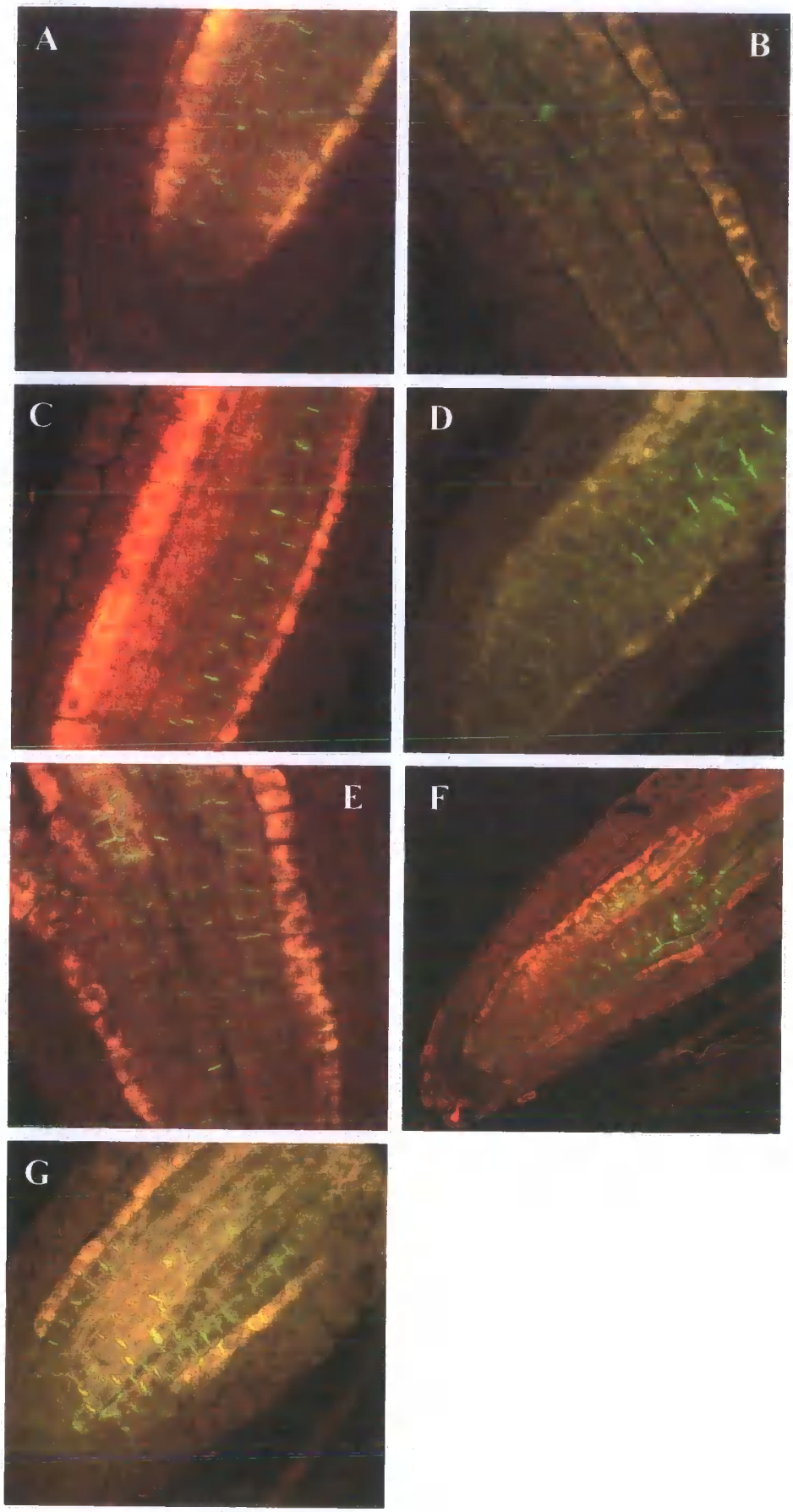


Figure 6.11.2

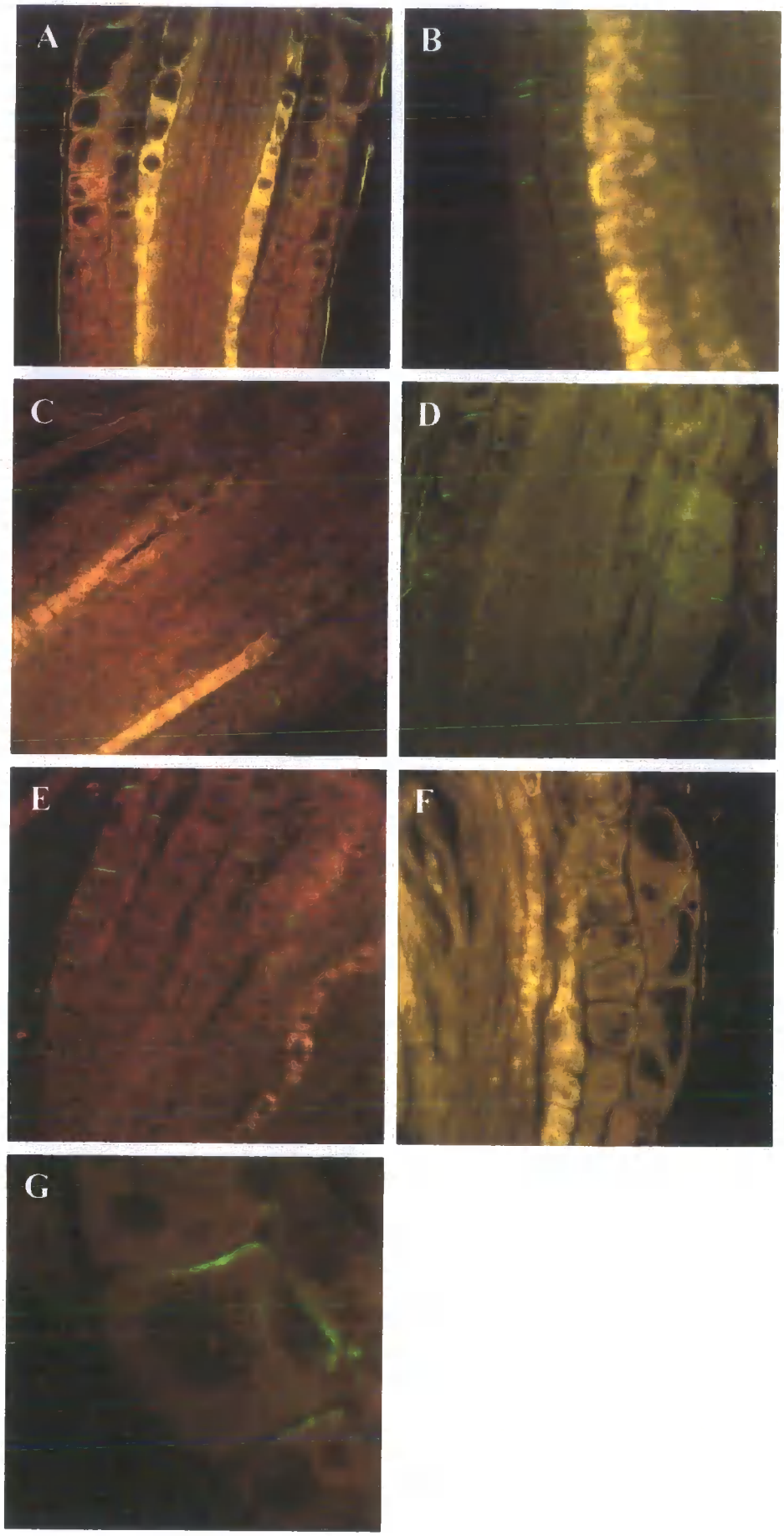
PIN2 Immunolocalisation

A and B: Localisation of PIN2 in C24.

C and D: Localisation of PIN2 in *hydra1*.

E, F and G: Localisation of PIN2 in *hydra2*.

Figure 6.11.2



In 6 and 12 day old *hydra1* and *hydra2* seedlings, both *PIN1* and *PIN2* were localised in the correct tissues and in the correct orientation within the cell. Figure 6.11.1 shows that *PIN1* was localised at the basal end of the cell throughout the vascular tissues in wild type plants as well as in both *hydra1* and *hydra2*. Figure 6.11.2 shows that *PIN2* was localised correctly at the apical side of the cell as well as the lateral wall of the cortex in wild type seedlings and in both *hydra1* and *hydra2* mutants.

These results demonstrate that the efflux carriers undergo correct trafficking and delivery to the cell membrane, as well as correct tissue expression.

6.12 The Auxin Efflux Carrier Protein PIN3 Shows Abnormal Localization in *hydra2* Mutants That is Rescued by Mutations in the *ETR1* Gene

A third PIN protein was immunolocalised in the *hydra* mutants. PIN3 protein is localised to the first tier of columella cells distal to the columella initials, on all faces of the cell (Friml *et al.*, 2002b). *pin3* mutants show a reduced amount of lugol staining in the columella because the differentiation of columella initials into columella cells (containing starch) is delayed. Also, the *DR5* maximum in *pin3* mutants is localised more distally in the columella of the root tip, suggesting that PIN3 helps to localise the maximum of auxin within the initials of the meristem, and therefore maintain their identity. PIN3 is therefore required for columella cell fate determination (Friml *et al.*, 2002b).

PIN3 localisation in *hydra1* was identical to wild type throughout development (Figure 6.12.1, A-F), and was normal even in roots that had undergone axis duplication (E and F). In *hydra2* however, this developmental staining pattern was not seen. At 6 days post germination, staining was as seen in the wild type (Figure 6.12.1, G and H), but by day 12 the expression of *PIN3* had moved up to the columella initials and the quiescent centre (I and J). By day 14 all *PIN3* expression had disappeared in the main root (K), but was still present in the lateral roots of the same plant (L). The timings of these observations are consistent with the loss of the columella root cap (3.13), already described, and the observation of a more distal shift in *DR5* expression before it is lost from the main root.

Figure 6.12.1

PIN3 Immunolocalisation

A and B: Localisation of PIN3 in C24.

C to F: Localisation of PIN3 in *hydra1* seedlings:

C: 6 days old.

D, E and F: 14 days old.

Figure 6.12.1

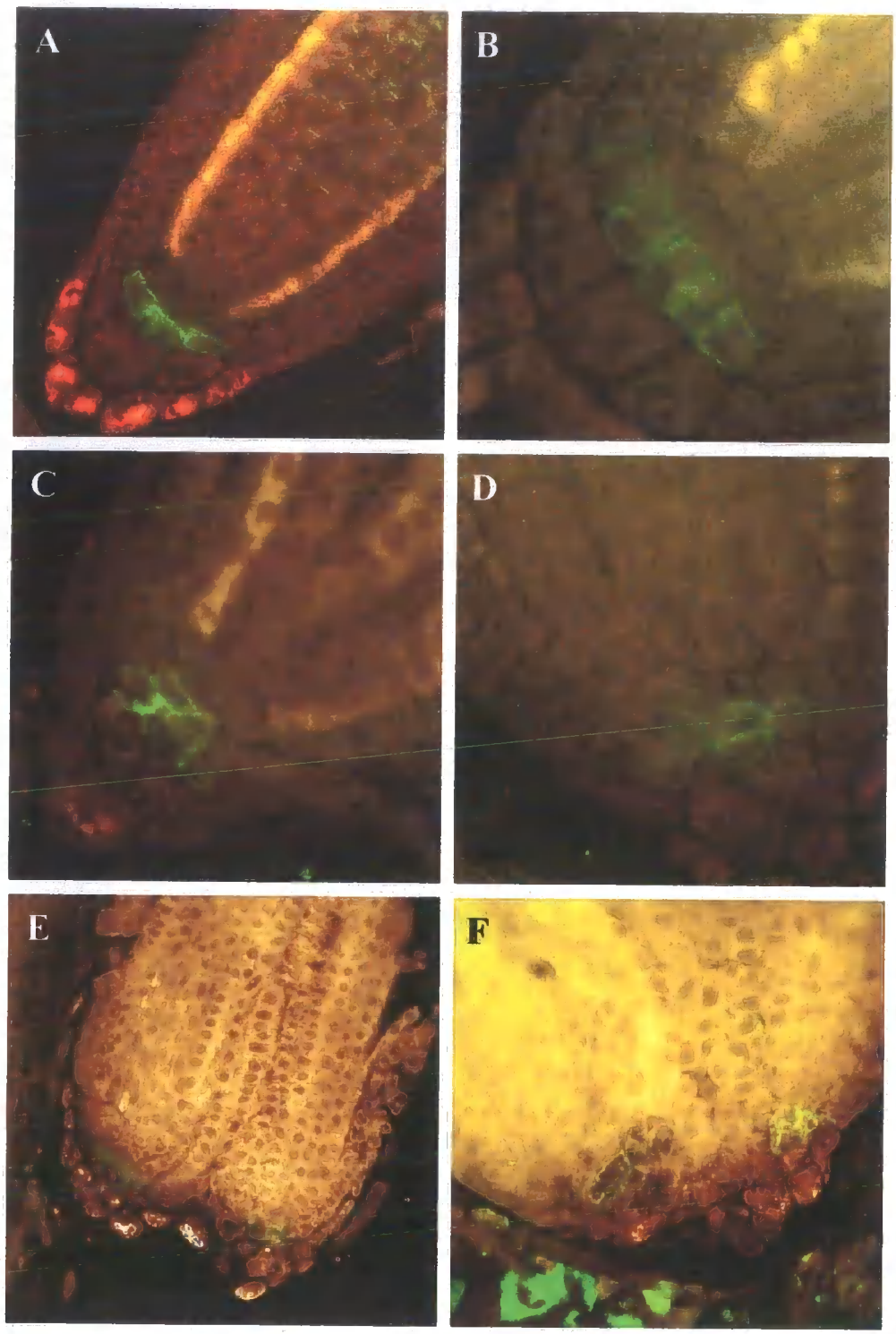


Figure 6.12.1

PIN3 Immunolocalisation

G to L: Localisation of PIN3 in *hydra2* seedlings:

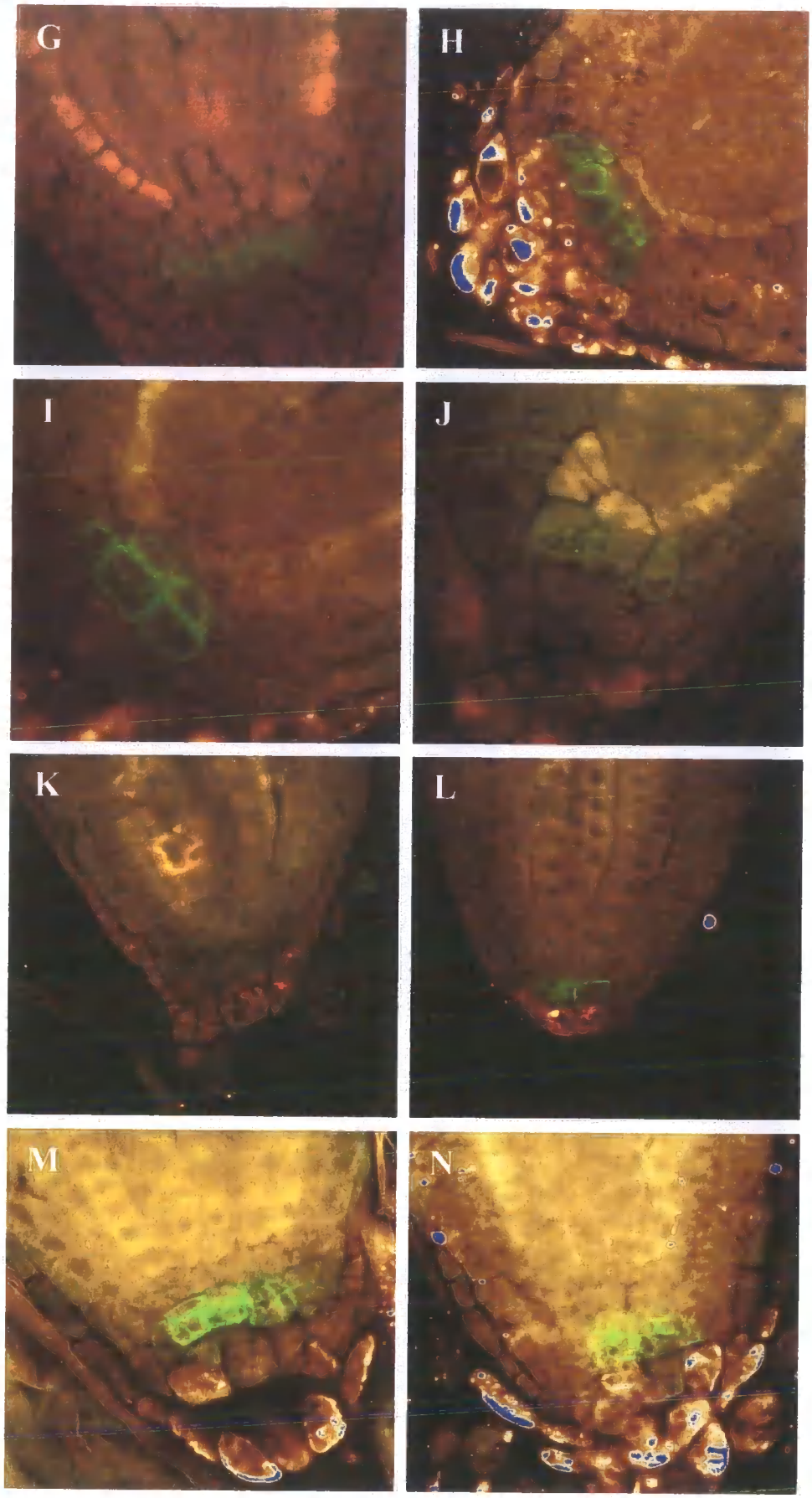
G and H: 6 days old

I and J: 12 days old

K and L: 14 days old

M and N: Localisation of PIN3 in *hydra2etr1-1* double mutants, 14 days after germination.

Figure 6.12.1



Previous experiments had implicated abnormal ethylene signalling in the cessation of cell division activity within the meristem and the loss of *DR5* staining within the root tip during this developmental time-frame. In order to investigate this further, PIN3 localisation was analysed in the root meristem of the putative *hydra2etr1-1* double mutants. *hydra2etr1-1* double mutants were stained with PIN3 antibodies 14 days after germination. The double mutants showed normal PIN3 localisation in the primary root at a time when it was absent within the *hydra2* primary root (see Figure 6.12.1).

These results show that there is no effect of either *hydra* mutation on the intracellular localisation of the *PIN* proteins. The tissue localisation of PIN1 and PIN2 was normal within both *hydra* mutants. However that of PIN3 was abnormal, being mis-expressed in adjacent cell layers and then being totally absent within the primary root. Expression is still seen in the lateral roots of the same seedlings at 14 days post germination, and the movement and loss of PIN3 in the main root correlates with the loss of the columella root cap at the same developmental stage. The more distal expression of *DR5* in *hydra2* before its loss also correlates with this staining pattern. Consistent with the rescue of root growth in the main root and the patterning of the columella root cap, inhibiting ethylene perception through the ETR1 receptor leads to a wild type and stable expression of PIN3.

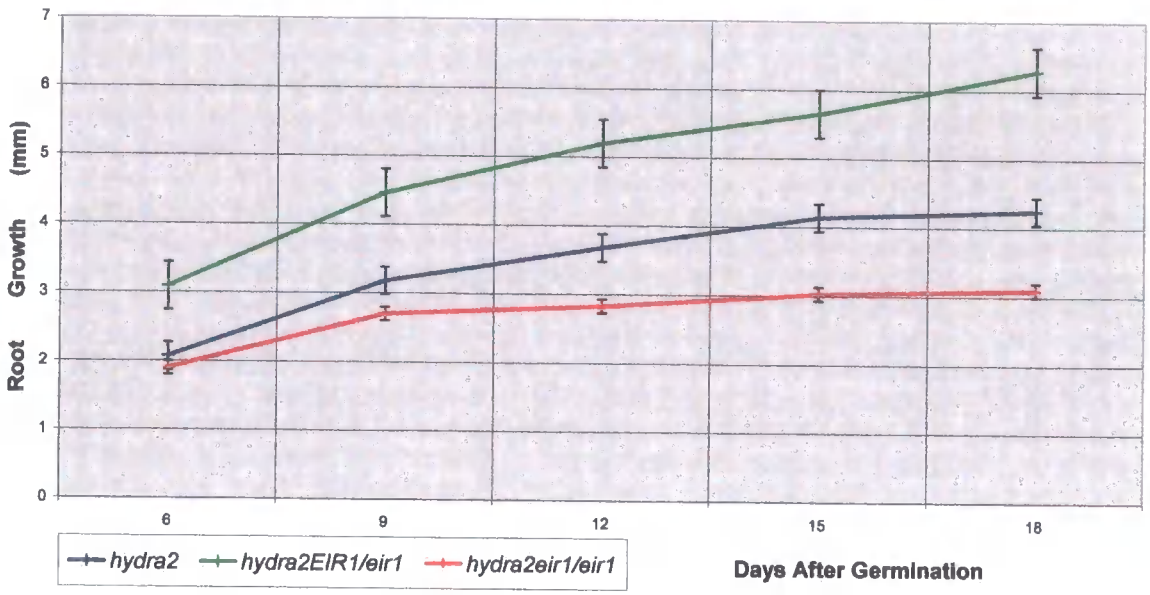
6.13 The Auxin Efflux Carrier Mutant *eir1* (*pin2*) is not Rescued in the *hydra2* Mutant Background

In order to determine if the same rescue of gravitropic response seen in the *aux1* mutants would occur in a strong agravitropic mutant allele of *PIN2*, *ethylene insensitive root1* (*eir1*) was crossed into the *hydra2* background (Muller *et al.*, 1998; Luschnig *et al.*, 1998). *eir1* double mutants were identified using a gravity response test identical to that used to isolate the *hydra aux1* double mutants (6.10). *hydra2eir1* double mutants were isolated from independent F2 lines that contained wild type agravitropic seedlings.

In order to measure the effect of the eir1 mutation on root growth, seedlings were germinated and grown vertically on the surface of half-strength MS medium. The primary root of the seedlings was measured every three days until 18 days post germination. 38 *hydra*-like seedlings were isolated from one F2 line containing

agravitropic *eir1* mutants. Three distinct populations were identified based upon the growth of the primary root. 23 out of 38 seedlings (approx. 2/4) had longer roots than *hydra2* mutants, which represents the predicted heterozygous *eir1* population. Of the remaining 15 seedlings, 6 seedlings had shorter roots than *hydra2* mutant seedlings (see Figure 6.13 below).

Figure 6.13 Root Growth in *hydra2eir1* Mutants



Gravity Response

Putative double mutants were subjected to a gravity response, as described in section 6.10 above, and failed to respond to the gravity stimulus (data not shown).

These results show that the *eir1* mutation caused an increase in root growth of the *hydra2* seedlings when in the heterozygous state, but caused a further reduction in root growth when in the homozygous state. The gravity defect of *eir1* is not rescued by the *hydra2* mutation.

6.14 Conclusion and Summary

The *hydra* phenotype is highly pleiotropic and differs between *hydra1* and *hydra2* only in the severity of the root phenotype. In *hydra2*, cell division within the root meristem diminishes and then ceases, a process that occurs at the same time as the root cap

columella is lost. Root hair patterning is also abnormal and contradicts the expression pattern of the *GL2* homeobox gene in the *hydra2* background.

The *HYDRA1* and *HYDRA2* genes encode sterol biosynthetic enzymes that, when mutated in the *hydra* mutants, cause gross changes in bulk sterol profiles. The mutants also lack BRs, however they cannot be rescued by their exogenous supply.

The mutants do not appear to be overproducing ethylene as inhibition of ethylene biosynthesis by AVG does not alter the *hydra* phenotype, but by inhibiting ethylene signalling *hydra2* root growth and cellular patterning can be rescued. *hydra2* also shows abnormal expression of a cytokinin up-regulated reporter gene, but fails to exhibit wild type ethylene-regulated feedback control upon the expression of the reporter.

The results presented in this chapter have shown that auxin signalling is also defective in the *hydra* mutants and contributes to certain aspects of the *hydra* phenotype. The *hydras* exhibit an increased sensitivity to exogenous auxins and show abnormal expression of two different auxin reporter genes, both of whose expression patterns can be rescued to wild type by inhibition of ethylene signalling. The intracellular localisation of three PIN proteins is similar to wild type. PIN3, however, shows abnormal tissue localisation followed by an absence of localisation, that correlates with the loss of the columella root cap, and can be rescued by inhibiting ethylene signalling. The influx carrier also shows abnormal behaviour in the *hydra* backgrounds. The agravitropic phenotype of two alleles of the influx carrier mutant *aux1* are rescued, and pharmacological inhibition of the influx carrier cannot prevent the ectopic up-regulation of an auxin reporter gene by the exogenously applied membrane-impermeable auxin analogue 2,4-D. Interestingly, *aux1-7* has a different effect in *hydra1* from *hydra2*, where it shows a semi-dominant phenotype and exhibits a greater root growth rescue than the *aux1-100* allele, which is only influential upon the phenotype in the homozygous state.

The *hydra* mutants provide the first direct evidence of a link between sterol biosynthesis and two major hormone signalling pathways. The aim of the final chapter of this thesis is to interpret and discuss the implications of these results, and to bring them together into a model to help explain the *hydra* phenotype.

Chapter 7

Discussion

Introduction

The aim of this chapter is to discuss the significance of the results presented in the previous four chapters, and to fit this information into a model that can explain the basis of the phenotype seen in the *hydra* mutants. I will firstly bring together all of the observations made about the root phenotype of the *hydra* mutants and discuss the most informative aspects. I will then examine the roles of the *HYDRA* genes in growth and development, and finally present a model for how mutations in these genes cause the numerous signalling defects that are evident in the *hydra* mutants.

7.1 The Highly Pleiotropic *hydra* Phenotype

The *hydra* phenotype is highly pleiotropic and differs between *hydra1* and *hydra2* only in the severity of the root phenotype. Both mutations cause defects in embryogenesis from the globular stage onwards, resulting in disoriented cell divisions that lead to a mature embryo that is stunted in the apical-basal axis but is radially swollen. Between three and six cotyledons develop at the shoot apex, that exhibits increased levels of cell division. The radial axis is often duplicated. It is possible that the defects in the shoot region are less amenable to rescue because they are established so early in development. Within the shoot region other defects are evident, such as in stomatal and vascular patterning, and abaxial-adaxial cell fate specification in the leaves.

7.1.1 Cell Division and Patterning Defects in the Primary Root

The majority of the work undertaken for this thesis focused upon the seedling root, which in contrast to the shoot has been more amenable to rescue. The hypophysis develops normally (Topping *et al.*, 1997), while the root meristem and hypocotyl, although present, do not elongate along the apical-basal axis. Both *hydra* mutants are morphologically identical at the end of embryogenesis. The post-embryonic root growth, however, differs between the two mutants. *hydra1* exhibited a short root that grew at a reduced rate when compared with wild type. *hydra2* had a short root that grew at a slower rate than *hydra1* before it ceased completely.

In *hydra2* seedlings cell division within the root meristem, as illustrated by the expression of the *CYCAT1:CDB:GUS* reporter gene and measured as primary root

growth, diminished and then ceased at ca. 18 days post germination. Cessation of cell division in the initials of the meristem is thus associated with the reduced root growth. Interestingly, staining with lugol failed to detect differentiated columella cells beyond ca. day 14, and so it is therefore possible that the reduction in cell division is also responsible for the loss of the patterning in the root tip. The QC is responsible for inhibiting the differentiation of the initials that surround it, and thus maintain their ability to divide and provide the cells required for the correct patterning and growth of the root (van den Berg *et al.*, 1995, 1997). If the QC is induced to follow a path of differentiation then this inhibiting signal is not produced and the initials subsequently differentiate rather than divide (van den Berg *et al.*, 1997). The loss of the root cap seen in the *hydra2* mutants could therefore be the result of the QC losing its identity, and the columella initials starting to differentiate into columella. If this was the case, then it would be expected that the root cap would be the first pattern element to be affected by a reduction in cell division, as the root cap initials generate the pattern as well as produce the cells to undergo differentiation. The initials behind the meristem feed cells into files of cells where the patterning signals come from more differentiated cells within the file (van den Berg *et al.*, 1995). The loss of lugol staining indicates that the differentiation process is reduced and then ceases, such that the initials assume the columella fate, but fail to undergo any further divisions. In support of this series of events, PIN3 localisation, which is normally in the first row of differentiated columella from the initials (Friml *et al.*, 2002b), had moved proximally one layer and was seen in the columella initials at ca. 12 days post germination. By ca. 14 days post germination expression had disappeared completely from the primary root. These observations correlate with the predicted series of events in which this pattern element would be lost. However, despite the fact that PIN3 has a role in columella cell fate specification (Friml *et al.*, 2002b), it is also possible that PIN3 is acting as a cell marker here, rather than as the mediator of the defect. PIN3 patterning in *hydra2* can be returned to wild type by the inhibition of ethylene signalling, indicating that ethylene is a major influence upon the activity of the primary root meristem in *hydra2*. This effect upon PIN3 localisation is restricted to the *hydra2* mutant, as PIN3 localisation in either ethylene overproducing or insensitive mutants is as wild type (J. Friml, personal communication).

7.1.2 The *hydras* do not Overproduce Ethylene

The most successful rescue of the *hydra2* root phenotype was achieved by inhibiting ethylene signalling, and so it was important to determine the exact point in the ethylene signal transduction pathway where the defect lays. To determine if the mutants overproduced ethylene they were grown in the presence of AVG, a known inhibitor of ACC synthases (Lieberman *et al.*, 1975; Tanimoto *et al.*, 1995). Chemical inhibition did not cause any rescue of the phenotype, indicating that the mutants were not overproducing ethylene, however, to confirm this actual ethylene levels were measured by Rachel Hackett and Don Grierson at the Plant Science Division, University of Nottingham, Sutton Bonington Campus, Loughborough.

Seedlings were grown *in vitro* to 7 days post-germination and evolved ethylene levels were then assayed (see Table 7.3.2). In two independent sets of experiments, the *hydra1* mutants (which are in a C24 background) produced ethylene at mean rates of 3.8 and 21.5nl ethylene g^{-1} FW h^{-1} respectively, compared with mean rates of 11.9 and 10.8nl ethylene g^{-1} FW h^{-1} for C24 wild types. The *hydra2* mutant (WS background) produced ethylene at mean rates of 15.5 and 6.3nl ethylene g^{-1} FW h^{-1}, compared to 16.7 and 12.0nl ethylene g^{-1} FW h^{-1}. This indicated that the mutants produced variable levels of ethylene, but did not produce dramatically different mean levels of ethylene compared to wild-type (Souter *et al.*, 2002).

Table 7.1.2 Ethylene Measurements
(nl ethylene g-1 FW h-1)

	C24	WS	*hydra1*	*hydra2*
	11.9	16.7	3.8	15.3
	10.8	12	21.5	6.3
Mean ± s.e.	**11.4 ± 0.8**	**14.4 ± 3.3**	**12.7 ± 12.5**	**10.8 ± 6.7**

This analysis showed that abnormally high levels of ethylene biosynthesis are not responsible for the observed phenotype. The possibility that the mutants produce locally high ethylene levels in specific tissues cannot be excluded, but the AVG results suggest that this is not the case.

When *hydra* seedlings were grown in the presence of silver ions, there was an increased rate of root growth and a rescue of the root phenotype in both mutants, but the effect was more dramatic in *hydra2*. This result indicated that the mutants may have an activated ethylene signal transduction pathway. Silver acts as a non-competitive inhibitor of ethylene binding, and was originally thought to associate strongly with the copper co-factor and inhibit the ethylene from binding (Beyer, 1976). However in ethylene binding assays in yeast it increases the affinity of the receptor for ethylene, suggesting that it acts by interfering with intramolecular signal propagation rather than with ligand binding (Rodriguez *et al.*, 1999). If the *hydras* were not rescued by silver then it would suggest that another element further downstream in the ethylene signal transduction pathway was defective. *CTR1* is the immediate downstream component from the receptor (Clark *et al.*, 1998), but *ctr1* mutants cannot be rescued by silver ions (Kieber *et al.*, 1993). Consequently, this suggests that the *hydra* mutants have an ethylene perception defect localised at the membrane-bound receptor.

To confirm the pharmacological evidence, a genetic mutation within the ethylene receptor *ETR1* was introduced into the *hydra2* background. Root growth recovery exceeded that achieved by the supply of silver ions, highlighting the importance of endogenous over exogenous manipulation of hormone pathways. Interestingly, a mutation in a gene downstream from the receptor in the transduction pathway, *EIN2*, caused a smaller amount of recovery in the root, but showed some recovery of the shoot. This suggests that the severity of the *hydra2* phenotype is due to ethylene receptor function.

Interestingly, *hydra2* root growth rate cannot be reduced any further by the addition of ACC, yet *hydra1* root growth can. It is therefore possible that ethylene signalling is effectively 'saturated' in *hydra2*, whereas in *hydra1* it is not saturated and so is able to respond and as a consequence it develops a more extreme phenotype in the presence of ACC.

7.1.3 Ethylene, Auxin and Cytokinin Influence Cell Division in the *hydra2* Root Meristem

As well as a defect in ethylene signalling, features of the *hydra* phenotype implicated defects in auxin signalling as well, such as abnormal vascular patterning (Mattsson *et*

al., 1999), the development of supernumerary cotyledons (Liu *et al.*, 1993), reduced root growth (Evans *et al.*, 1994), defective patterning of the root tip (Sabatini *et al.*, 1999), and defective root hair elongation (Pitts *et al.*, 1998). In order to investigate the influence of auxin on root growth, auxin- regulated GUS reporter genes were crossed into the *hydra* backgrounds, and double mutants were created with well-characterised auxin mutants.

The *hydra2* mutants showed abnormal expression of two different auxin-induced GUS reporter genes within the root meristem region. Both *DR5* and *IAA2::GUS* showed a wild type expression pattern until ca. day 12-15, when there was a reduction in staining in the primary root meristem, that was followed by a complete loss of expression in the primary root tip by ca. day 18. The timing of the reduction and loss of expression of these two reporter genes overlapped with the observed reduction in cell division (*CYCAT1:CDB:GUS* expression), and the loss of the root cap columella. By contrast *hydra*1 showed wild type expression throughout development. This therefore links abnormal auxin responsiveness with the cessation of cell division and loss of patterning in the primary root of *hydra2*. However it is imperative that we separate cause and effect, and in this case the wild type expression of both reporter genes in *hydra2* was rescued by inhibiting ethylene signalling with silver ions.

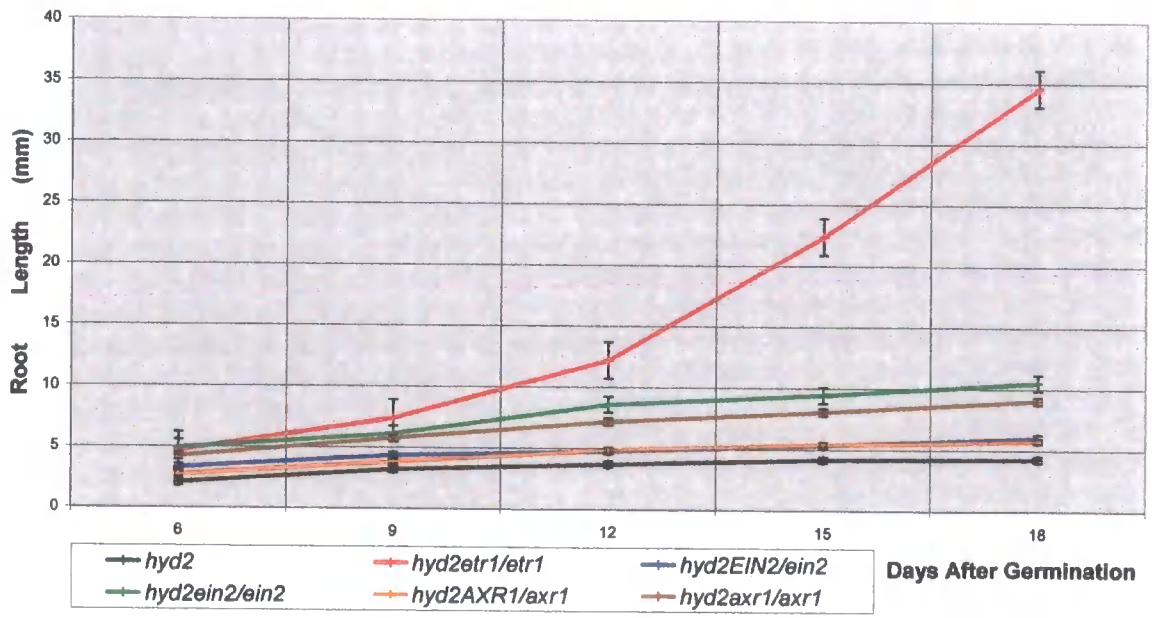
In order to manipulate auxin responses endogenously, genetic crosses were made with the auxin resistant *axr1-12* and *axr3-1* mutants. *AXR1* encodes a subunit of the auxin-stimulated RUB-activating enzyme complex that activates the SCF^{TIR1} complex, that ubiquitinates the transcriptional repressor AUX/IAA proteins, targeting them for degradation, thus allowing transcription of auxin-induced genes (Leyser *et al.*, 1993; Tiwari *et al.*, 2001). *AXR3* encodes one of the AUX/IAA transcriptional repressor proteins, IAA17, and the domain II mutation, the site of ubiquitination, in *axr3-1* causes a 7-fold increase in the stability of the protein (Ouellet *et al.*, 2001).

axr1-12 has a longer root than wild type seedlings, and in both *hydra* backgrounds the mutation resulted in a longer root. There was a more dramatic increase in root length in *hydra2* than in *hydra1*, as well as a heterozygous *axr1-12* phenotype in the *hydra2* background. These results indicate that a defect in auxin signalling may contribute to the root meristem phenotype of *hydra2*, as reducing the level of responsiveness of the seedling by half (in the heterozygous *axr1-12 hydra2* mutants) caused an increase in

root growth. However the influence of ethylene cannot be ruled out, as *axr1* mutants exhibit some ethylene insensitivity. Initially this was thought to be due to an inability to activate ethylene biosynthesis via an auxin-inducible ACC synthase (Lincoln *et al.*, 1990). Recent work on a number of different auxin mutants has led to the proposal that ethylene signalling in the root requires a certain cellular level of auxin within the proximal and distal elongation zone. The effect of the *aux1* and *eir1* mutations (in the influx and efflux carriers respectively) is to reduce the amount of auxin within the elongation zone of the root (Swarup *et al.*, 2001; Luschnig *et al.*, 1998), whilst the effect of the *axr1* and *axr3* mutations is to reduce the amount of auxin responsiveness within these tissues (Leyser *et al.*, 1996). However, although the induction of genes with AREs within their promoters could include an ACC synthase, there is currently no evidence to further this hypothesis.

The recovery of root growth by the *axr1-12* mutation needs to be put in the context of the effects of ethylene. Figure 7.1.3 shows *hydra2* root growth in the *ein2*, *axr1-12* and *etr1-1* double mutants.

Figure 7.1.3 *hydra2* Double Mutant Root Growth



There was a similar level of root growth in the *hydra2ein2* and *hydra2axr1-12* double mutants, which both had effects in the heterozygous condition. However, in comparison the *hydra2etr1-1* double mutants showed a considerable recovery of root growth that was greater than the other double mutants. This could be due to the effect of the *ein2*

and *axr1-12* mutations upon other signalling pathways, where they are part of hormonal crosstalk (Alonso *et al.*, 1999; Leyser, 1998; Lincoln *et al.*, 1990). *ein2* mutants show high ethylene insensitivity, but of the 25 alleles isolated so far, some were found in screens for resistance to either auxin transport inhibitors, cytokinin or ABA. No other loci connected with ethylene signalling have been isolated in these screens, suggesting that it is also a central component of other signalling pathways (Alonso *et al.*, 1999). As already mentioned, *axr1* mutants also show some ethylene insensitivity (Leyser, 1998; Lincoln *et al.*, 1990).

It is therefore possible that the *etr1-1* mutation causes the greatest increase in root growth, when compared with the *ein2* and *axr1-12* mutations, because it only affects ethylene signalling. The *axr1-12* mutation reduces not only auxin sensitivity but also sensitivity to ethylene. When *ein2* is mutated then *hydra2* not only develops reduced ethylene signalling, but it also develops reduced responses to other hormones that use it as part of their signalling pathway, and it is this that results in the reduced amount of recovery when compared with the *etr1-1* mutation.

This implicates a role for both the *EIN2* and the *AXR1* gene products in the (greater) level of root growth recovery seen in the *hydra2etr1-1* double mutant. Or more specifically, auxin sensitivity is required for the most optimal rescue of root growth in *hydra2*, provided that ethylene insensitivity has been achieved at the membrane-bound receptor.

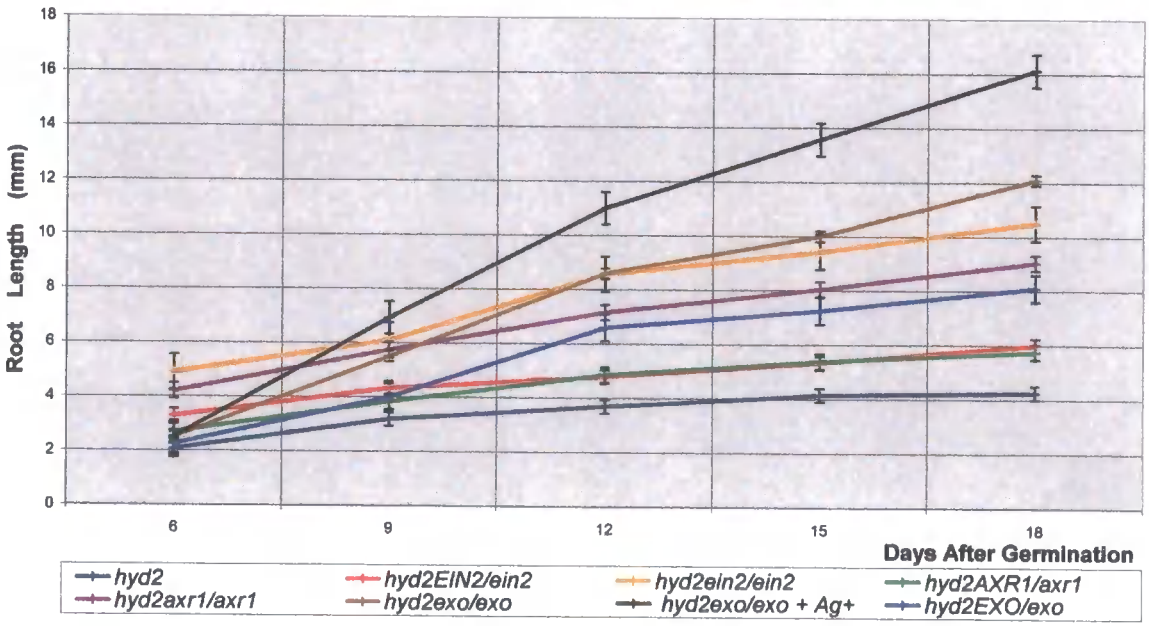
7.1.4 Mutations in the Auxin-Induced *EXORDIUM* Gene Rescue Root Growth in *hydra2*

Although ethylene is the major influence upon the activity of the *hydra2* root meristem, auxin responsiveness is also involved. The rescue of *hydra2* root growth by mutations in the *EXORDIUM* (*EXO*) gene gives further insight into this relationship. Studies of the *EXO* gene, which was tagged by a promoter trap T-DNA, have revealed that the expression of this cell cycle phase-dependent gene in the root meristem may be influenced by auxin (Farrar *et al.*, submitted). The *EXO* promoter contains the TGTCTC sequence present in AREs, and so offers a mechanism through which the position of expression within the meristem is achieved - auxin levels are greatest within the QC and initials of the meristem (Sabatini *et al.*, 1999; Kerk and Feldman, 1995). In support of

this, inhibition of auxin influx (with 1-NOA) or efflux (with NPA) causes a shift in the activity of the *EXO* gene promoter. As well as being up-regulated by auxin the *EXO* gene is down-regulated by cytokinin, and since cytokinins are synthesised in the root tip (Sossountov *et al.,* 1988; Chiapetta *et al.,* 2001), they might be expected to limit the level of *EXO* expression in the meristem. Therefore *EXO* expression would appear to be antagonistically regulated by both auxin and cytokinin within the meristem (Farrar *et al.,* submitted).

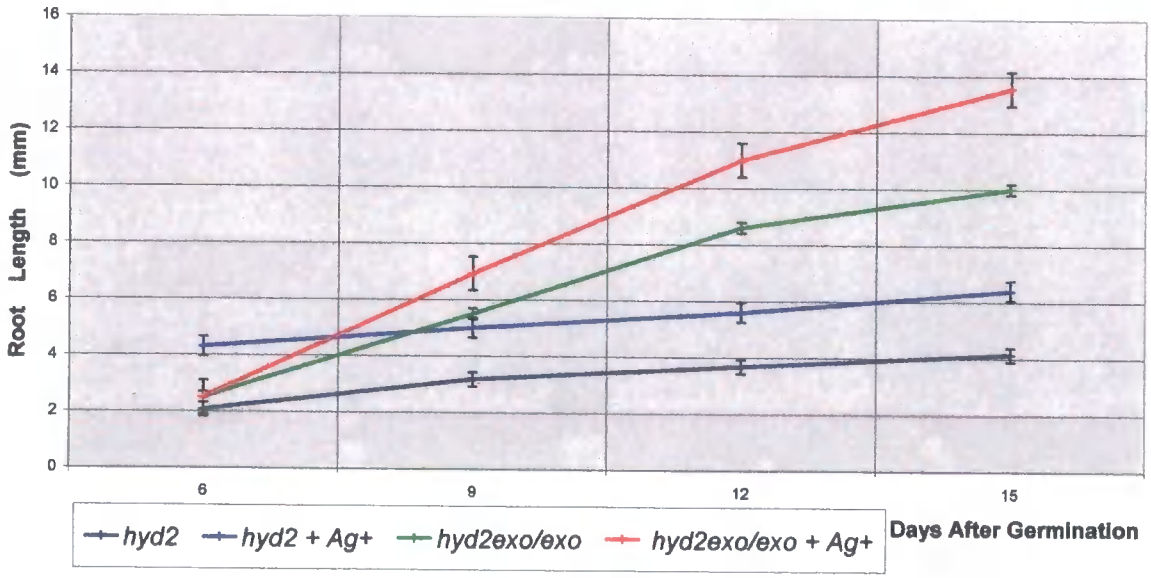
The mutation in the *EXO* gene in the promoter trap line AtEM201does not completely ablate expression of the *EXO* transcript, and instead less than half wild type levels are produced. No obvious mutant phenotype is seen in the AtEM201 line or in transgenic antisense lines, and since it is a member of a small gene family of structurally related genes in *Arabidopsis*, it may be functionally redundant. However, a reduction in *EXO* expression in the *hydra2* mutant background led to a significant rescue of *hydra2* root growth, and the predicted *hydra2EXO/exo* double mutants showed an intermediate level of recovery when compared with *hydra2exo/exo* double homozygous mutants. This is similar to the *axr1-12* mutation, which also had an effect upon root growth in *hydra2* in the heterozygous state. However, when the recovery in the *axr1*, *ein2* and *exo* mutant backgrounds are compared, the *exo* mutation caused an even greater increase in growth rate than either *axr1-12* or *ein2-1* (Figure 7.1.4.1).

Figure 7.1.4.1 Comparison of *hydra2* Root Growth Recovery by the *axr1-12*, *ein2-1* and *exo* Mutations



These results suggest that the *exo* mutation suppresses some of the ethylene effects upon the root tip. One possibility is that *EXO* functions as a negative regulator of cell division, and its transcriptional suppression by cytokinin would be consistent with the cell cycle-promoting effects of this hormone (Riou-Khamlichi *et al.,* 1999). Suppression of the effects of *EXO* in the meristem would therefore be required to promote cell division, and this is consistent with the observation that reducing the level of *EXO* expression (as in the AtEM201 line) increases cell division within the *hydra2* root meristem. This means that both auxin and cytokinin are key influences in creating the *hydra2* root meristem phenotype, but how does this relate to the observed role of ethylene? In support of the comparative evidence, silver ions have an additive effect upon the *exo*-mediated rescue of the *hydra2* root, indicating that both *exo* and silver ions are working in separate pathways (Figure 7.1.4.2). However, before we can explain how these hormones might interact, we must look closer at the influence of cytokinin.

Figure 7.1.4.2 *hydra2* Root Growth Recovery With Silver Ions and the *exo* Mutation



7.1.5 Hormonal Crosstalk: *ACS1::GUS* Expression and Auxin, Cytokinin and Ethylene

ACC synthases are the rate limiting enzyme in the ethylene biosynthetic pathway (Liang *et al.,* 1995). Some members of the ACC synthase gene family are specifically responsive to hormones, and both auxin and cytokinin have been implicated in acting through ethylene and regulating the levels of ACC synthases (Vogel *et al.,* 1998). Ethylene also positively feeds back on its own biosynthetic enzymes.

ACS1::GUS is a developmentally and hormonally regulated reporter gene, and although it is a pseudo ACC synthase, lacking essential residues for catalysis of the conversion of *S*-adenosyl methionine to ACC (Liang *et al.*, 1995), it acts as a marker to allow an understanding of the internal balance of different hormones at specific time points in development. In the wild type root it is expressed in the endodermis and vascular tissue, and is up-regulated by cytokinin and ethylene (Rodrigues-Pousada *et al.*, 1999).

In *hydra1*, *ACS1* expression was wild type throughout development, whilst in *hydra2* it was wild type at ca. 3 days post germination, but at ca. day 6 was massively up-regulated throughout all cell layers and into the root tip itself, where it is not normally expressed. By ca. 9 days post germination, and for the rest of development, this ectopic expression had reverted back to wild type again. This difference in expression reflects the differences in root growth between *hydra1* and *hydra2*, and therefore can be interpreted as being connected with the events that take place within the *hydra2* root meristem.

Initially, this temporal and spatial expression pattern suggested that either ethylene or cytokinin up-regulated *ACS1::GUS* expression between days 4-6, and that this influence was reduced to wild type levels between days 7-9. To determine the influences of these hormones in the *hydra2* background, *ACS1::GUS* expression was analysed in *hydra2* mutants grown on ethylene (ACC) or cytokinin (BA) sources. Expression could not be induced by ACC, which was in conflict with the wild type, where an increase in expression was seen (Rodrigues-Pousada *et al.*, 1999). BA, on the other hand, could induce expression, and even caused a phenocopy of the expression pattern seen in *hydra2* seedlings on ca. day 6. This suggested that cytokinin was responsible for the up-regulation of the reporter gene ca. 6 days post germination. It is also possible that *hydra2* is insensitive to exogenous ACC, since *ETR1* may not function properly. Such a possibility will be discussed in more detail later.

In wild type seedlings, *ACS1::GUS* expression is down-regulated by auxin, and this response was wild type in both *hydra* mutants. So it is therefore possible that auxin is responsible for the down-regulation of the *ACS1* promoter between ca. 7 and 9 days post germination. This observation is consistent with measurements taken of the levels and movement of shoot-derived auxin upon the levels of auxin in the primary root during this time frame of development (Bhalerao *et al.*, 2002). Between ca. 5 and 7 days

post germination, the first leaves start to develop at the shoot apex, and they immediately become net-exporters of auxin. As well as the first leaves, which are the major source, the shoot meristem and the cotyledons also contribute to a release of auxin, in the form of a pulse or a wave, that leaves the shoot at ca. days 5-7 and reaches the root tip between days 7-9 (Bhalerao *et al.*, 2002). The timing of the release of the auxin wave and its arrival in the root tip coincides with the down-regulation of the *ACS1::GUS* reporter. This could therefore account for the reduction in the expression of the reporter, as the arrival of the auxin wave would restore the wild type balance of cytokinin to auxin within the root tip.

The *hydras* have multiple cotyledons and initiate multiple first leaf primordia. It can therefore be envisaged that a larger-than-wild type amount of auxin is present in the wave. This could explain the *axr1-12* and *exo* results, in which a reduction in auxin response can help to alleviate the reduction in cell division that starts to occur after the arrival of the auxin wave. Unfortunately, without direct measurements of the levels of cytokinin and auxin in the primary root over this time period, it is difficult to speculate further with any confidence. At the time of writing measurements of auxin and cytokinin levels are being performed by GARNET.

7.1.6 Interpreting *EXORDIUM* Function and the *hydra2* Root Meristem Phenotype

EXO is down-regulated by cytokinin, which in *hydra2* may have a predominant influence in the root tip until ca. day 7 post germination, when auxin from the shoot reaches the root tip and redresses the balance. If *EXO* works as a negative regulator of the cell cycle, then it would be expected that reducing the levels of *EXO* mRNA would cause an increase in root length. This is what is observed in the *hydra2* root. It is therefore possible that cytokinin synthesised in the wild type meristem suppresses high levels of *EXO* transcription to allow cell division to proceed. A consequence of relatively high cytokinin levels at the meristem is an increase in ethylene levels, such that many cytokinin responses may in fact be mediated by ethylene (Vogel *et al.*, 1998). However, in *hydra2* we have already implicated ethylene as a major influence upon the meristem, and that this may be due to a defect at the receptor, not ethylene biosynthesis. Consistent with this is the finding that inhibiting ethylene signalling with silver ions leads to an enhancement of the recovery of *hydra2* root growth initiated by the *exo*

mutation. Indeed, down-regulation of *EXO* levels by cytokinin (or by the *exo* mutation) may have the effect of reducing cellular responses to ethylene that may be induced by cytokinins (Vogel *et al.*, 1998). However in the *hydra2* mutants the application of exogenous cytokinin does not rescue root growth. Certainly a role for the *exo* mutation in reducing the impact of high cytokinin levels cannot be excluded, but it appears that an alternative explanation for the enhanced recovery of root growth is required.

The reduced level of *EXO* may in fact play a more important role later, after the cytokinin influence has passed. The subsequent effect of the shoot-derived auxin accumulating at the root tip must be taken into account. If auxin induces expression of *EXO*, and it does work as a negative regulator of the cell cycle, then it must, in the *hydra2* mutant, result in a considerable reduction in the cycling of the dividing cells in the meristem, the initials. The corollary of this is that in *hydra2exo* double mutants, the reduced amount of *EXO* transcript allows cell division to continue despite the cell cycle-inhibitory levels of auxin that are accumulating at the root tip. This is a satisfactory explanation, as the *axr1-12* evidence confirms a role for auxin in the reduction of root growth in the *hydra2* mutant.

7.1.7 Ethylene has a Negative Effect Upon the Cell Cycle

As has been suggested, if the QC and the initials lose their ability to divide and start to differentiate, then it will have profound implications for the patterning and growth of the rest of the primary root. Both auxin and cytokinin have well documented links with cell division (see section 1.4.1), however it appears that ethylene has an over-riding effect on cell division within the root meristem of the *hydra2* mutants. So what evidence is there of a direct interaction with the cell cycle?

For many years a role for ethylene in cell cycle control was unclear. However recent work has shown that it does have an influence, although it is not a promotive one. When cultures of aphidicolin-synchronised Tobacco BY-2 cells were supplied with gaseous ethylene the cells arrested at the G2/M checkpoint before undergoing apoptosis (Herbert *et al.*, 2001). This effect was ameliorated by the addition of silver ions, showing that the effects are mediated through the ethylene receptor. Nuclear shrinkage and DNA fragmentation were characteristics of dying cells at G2/M, suggesting a programmed mechanism of cell death exacerbated by ethylene treatment (Herbert *et al.*, 2001).

These findings provide a cellular basis for the effect of ethylene upon the *hydra2* root meristem. The *hydra2* mutants do not overproduce ethylene, but because of a defect at the ethylene receptor the mutants induce ethylene responses as if they are perceiving a large amount of it. The main effect of this appears to be upon the QC and the initials that surround it, which cease dividing and instead differentiate, and in the case of the columella initials this means that they start to express PIN3. This loss of patterning combined with a reduced number of cells entering the zone of elongation means that root growth ceases, and the expression of the *DR5* and *IAA2::GUS* reporter genes is disrupted.

It can therefore be proposed that if ethylene signalling is inhibited at the receptor then cell division continues in the initials and the QC maintains its identity. The root cap would still be produced through the continued cell divisions of the columella initials, which would maintain their undifferentiated identity because of signalling from the QC inhibiting their differentiation (van den Berg *et al.*, 1997). The first row of differentiated columella would thus continue to express PIN3. The consequence of this is the maintenance of the auxin transport stream running through the root tip (Friml *et al.*, 2002a), and thus the correct spatial expression of *DR5* and *IAA2::GUS* within the root meristem and elongation zones.

Continuing this speculative hypothesis, if *EXO* is down-regulated by a high level of cytokinin at ca. 6 days post germination, then it would allow the cell cycle to progress unimpeded. However once the pulse of auxin reaches the root tip at ca. days 7-8 post germination, it would cause the down-regulation of the *ACS1::GUS* reporter gene and the up-regulation of the *EXO* gene. *EXO*, acting as a negative regulator of division, starts to slow down the cell cycle, which could make the dividing cells more susceptible to differentiation because of the effect of ethylene. The *axr1-12* mutation would therefore reduce the influence of auxin, possibly through the level of *EXO* transcription, and thus encourage cytokinin to increase root growth through cell division.

The effect of the *axr1-12* and *exo* mutations upon *hydra2* root growth could therefore be due to the increased amount of cell division, which in the *hydra2* mutant may be caused by auxin-induced *EXO* suppression of the cell cycle.

Because the inhibition of ethylene signalling with silver ions caused an additive increase in root growth in the *hydra2exo* double mutants, it suggests that the *exo* mutation does not completely suppress ethylene effects, and may act either partially through the inhibition of ethylene action, or independently of it, perhaps via the inhibition of ethylene-independent auxin effects at the root tip. Therefore, *EXO* may be a component of a mechanism by which meristem cells interpret auxin, cytokinin and ethylene signals, to regulate cell division responses.

Summary

Defective ethylene signalling predominates in creating the *hydra2* root phenotype, but defective auxin and cytokinin signalling are also involved in adding to its complexity. An essential question then, is how do these numerous signalling defects relate to the role of the *HYDRA* genes?

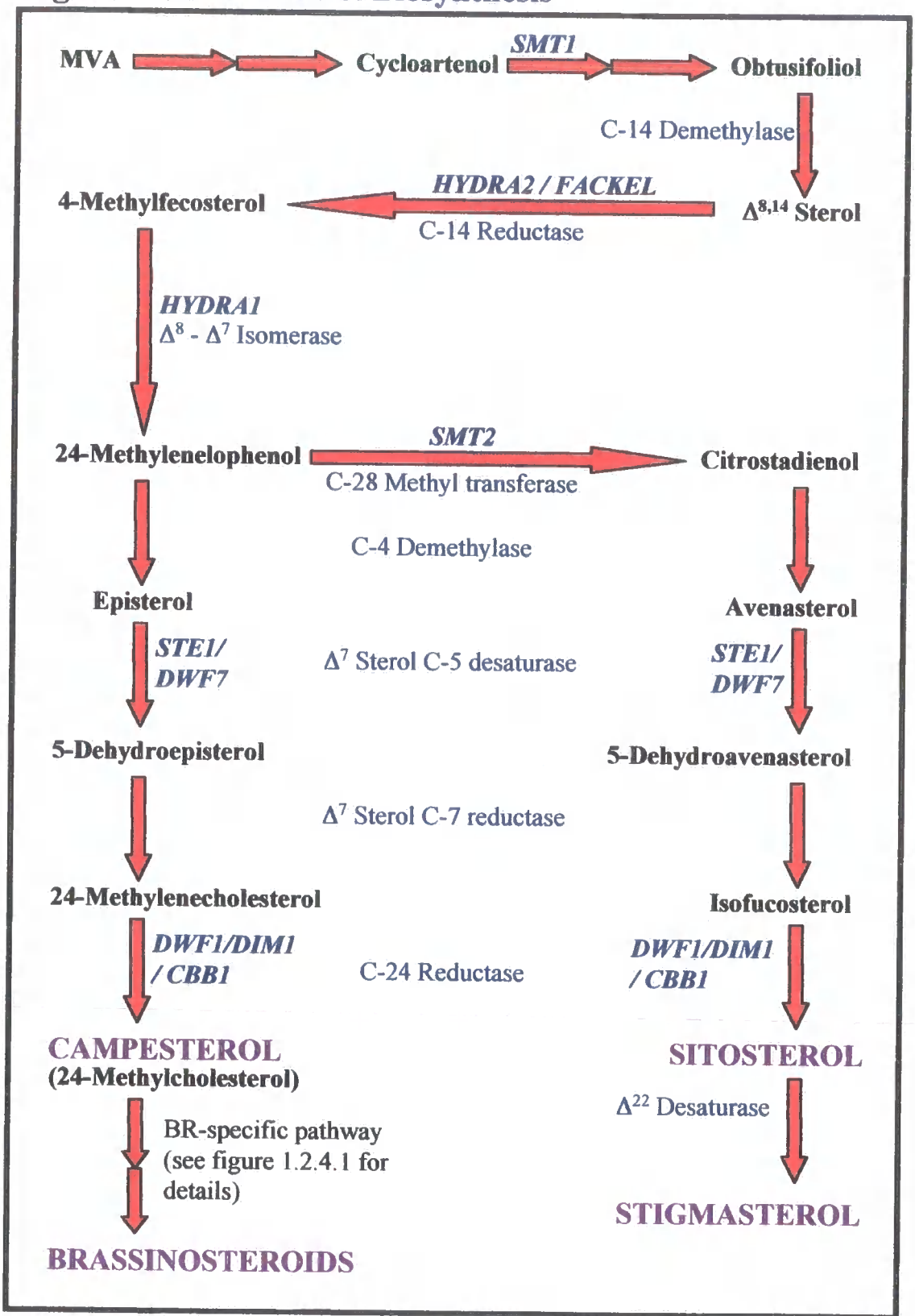
7.2 The *hydras* are Sterol Biosynthetic Mutants

The two *hydra* mutants are defective in the expression of two genes encoding different sterol biosynthetic enzymes. *HYDRA2*, which is allelic to *FACKEL*, encodes a C-14 sterol reductase, whilst *HYDRA1* encodes a Δ8-Δ7 sterol isomerase, one step downstream of the sterol reductase within the sterol biosynthetic pathway. Fig. 7.2 shows the position of both *HYDRA* genes within the sterol biosynthetic pathway.

7.2.1 *HYDRA2/FACKEL* Encodes a C-14 Sterol Reductase

The *HYDRA2/FACKEL* gene encodes the sterol biosynthetic enzyme C14 sterol reductase (Jang *et al.*, 2000; Schrick *et al.*, 2000). The gene consists of 13 exons and 12 introns, and is situated on chromosome III. It makes a conceptual protein of 369 amino acids with a predicted molecular mass of 41-42 kD, is highly leucine-rich (16.5%) and almost entirely hydrophobic (Jang *et al.*, 2000; Schrick *et al.*, 2000). Hydropathy plot analysis suggests that *HYDRA2/FACKEL* encodes a membrane-bound protein with eight to nine transmembrane domains and a C-terminal cytosolic tail of about 43 amino acids. Computer analysis predicts an uncleaved N-terminal signal sequence and protein localisation to the endoplasmic reticulum or plasma membrane (Jang *et al.*, 2000;

Figure 7.2 Plant Sterol Biosynthesis



Key: Enzymes catalysing these reactions and ***Arabidopsis* genes corresponding to these enzymes.** Multiple arrows represent more than one biosynthetic step not included here. Taken from Schrick *et al.*, (2000) and Jang *et al.*, (2000).

Schrick *et al.*, 2000). *FACKEL* cDNA complements the growth defect of yeast *erg24* cells (Schrick *et al.*, 2000), confirming a sterol reductase function for this protein.

In situ hybridisation studies show *FACKEL* mRNA is detectable in the early stages of embryogenesis, in a uniform staining pattern throughout globular and early heart stage embryos. However, at the mid-heart stage the signal becomes restricted towards the top and bottom of the embryo, and in the mature embryo it is confined to the shoot and root meristems (Schrick *et al.*, 2000). To confirm this a *FACKEL::GUS* reporter gene transgenic line showed expression predominantly in areas where cell divisions occurs (Jang *et al.*, 2000). These results show that the protein is made to high levels within these tissues, and this is to be expected because of the rapid amount of cell division and the subsequent requirement for new cell membranes.

The wild type expression pattern of the gene correlates with the mutant embryonic phenotype and with the defects in the meristems seen in post-embryonic development. RT-PCR analysis of the *hydra2* mutant showed no detectable mRNA transcript, indicating that the mutation has caused a null allele, possibly due to a T-DNA insertion event.

7.2.2 *HYDRA1* is a Δ8-Δ7 Sterol Isomerase

A genomic fragment of 2.6 kb flanking the T-DNA in *hydra1-2* was cloned by plasmid rescue and found to be 100% homologous to the 5' region of an EST which was identified as a cDNA encoded by the *Arabidopsis* Δ8-Δ7 sterol isomerase gene (Souter *et al.*, 2002). The gene is situated on chromosome I. The *HYDRA1* protein has four predicted membrane spanning domains and a consensus ER-retrieval domain at the C-terminus. The T-DNA was found to have inserted into the first intron of the gene, which most likely results in a null mutation, and this is supported by the observed lack of detectable *HYDRA1* transcript in RT-PCR analysis (Souter *et al.*, 2002). The *Arabidopsis* sterol isomerase had been cloned previously and demonstrated to have sterol isomerase enzyme activity (Grebenok *et al.*, 1998), however no corresponding developmental mutant phenotypes had previously been identified in yeast, plants or animals.

A *HYDRA1* promoter-GUS fusion was expressed at the globular and heart stage and was constitutive in the embryo-proper but not detectable in the suspensor, which is consistent with the observed defects being restricted to the embryo proper from the globular stage onwards, and the correct development of the suspensor. Torpedo stage embryos showed lower levels of GUS activity, whilst cotyledonary and mature stage embryos had virtually no detectable GUS expression (Souter *et al.*, 2002).

7.2.3 Sterol Analysis Confirms a Role For These Genes in Sterol Biosynthesis

An analysis of the sterol levels by the commercial Lipid Analytical Service at the Scottish Crops Research Institute, Dundee, showed gross changes in the ratios of bulk sterols (see Chapter 4, Table 4.2).

Both *hydra1* and *hydra2* had much reduced levels of sitosterol and campesterol, with elevated levels of stigmasterol, consistent with the levels reported for *fackel* (Jang *et al.*, 2000; Schrick *et al.*, 2000). These results confirm the role of these genes in sterol biosynthesis, and demonstrate that mutations in the sterol isomerase (in *hydra1*) and the sterol reductase (in *hydra2/fackel*) result in similar gross changes in sterol profiles in *Arabidopsis*.

The sterol biosynthetic pathway in plants can essentially be viewed as containing two parts, the first produces the bulk membrane sterols and the precursors of the BR pathway, whilst the second part contains the BR-synthesis branch. Figure 7.2 shows a summary of the sterol pathway and the position of the *HYDRA* genes within it, as well as those of other genes known in *Arabidopsis*.

7.2.4 Other Sterol Biosynthetic Mutants and Their Phenotypes

A number of mutants in plant sterol biosynthetic genes have been identified, most of which, until recently, were found to encode enzymes required for the synthesis of BRs. Such mutants are typically dwarfed and can be phenotypically rescued by exogenous BR application (e.g. Szekeres *et al.*, 1996; Choe *et al.*, 1999a, b). However, both *HYDRA* genes are high in the pathway, acting before the split towards campesterol and BR production or sitosterol and stigmasterol, and the sterol profiles of the mutants reflect this. *hydra1* has 12% wild type levels of campesterol, 2% sitosterol, but an

accumulation of stigmasterol, 182% of wild type levels. *hydra2* however, lacked campesterol (0%), but had a slightly higher level of sitosterol (4%) and a massive accumulation of stigmasterol (322%). Interestingly, the *hydra2* mutants are deficient in BRs but cannot be rescued by their exogenous application (Topping *et al.*, 1997; Jang *et al.*, 2000; Schrick *et al.*, 2000), and a possible reason for this will be discussed later.

The proximity of the *HYDRA* enzymes in the pathway presumably accounts for the similarities in the sterol profiles of the two mutants as well as the similar phenotypes. However, the differences in sterols (the *hydra2* mutants accumulate considerably more stigmasterol and totally lack campesterol), may therefore account for the observed developmental differences between the mutants.

A number of papers have recently been published exploring the effect of up-regulating or down-regulating some of the genes encoding enzymes within the sterol pathway, linking the resultant changes in sterol profiles with numerous growth defects. This therefore allows us to interpret the influence of the different bulk sterols upon growth and development, and which can help in identifying the impact of the *hydra* sterol profiles.

sterol methyltransferase1 (*smt1*) mutants accumulate cholesterol at the expense of sitosterol, whilst levels of campesterol are unchanged (Diener *et al.*, 2000). The mutants are short, have reduced fertility, and show reduced expansion in the petiole and leaf blade, resulting in small round leaves. Root growth is stunted as a result of reduced cell division and cell expansion, and root hair patterning is disorganised, possibly due to swellings in the cortex and endodermis causing a delineation of the cells. Although the mutants are hypersensitive to BRs, they cannot be rescued by them. An *SMT1::GUS* promoter construct shows expression predominantly within cells undergoing expansion (Diener *et al.*, 2000).

Transgenic tobacco plants expressing a *35S::STEROL METHYLTRANSFERASE2* (*SMT2*) construct accumulate sitosterol at the expense of campesterol, causing reduced stature and growth, that can be rescued by BRs (Schaeffer *et al.*, 2001). Co-suppression of *SMT2* causes low sitosterol and high campesterol generating pleiotropic defects such as reduced growth and low fertility, which is not modified by BRs. However the

reduced growth was due to a reduction in cell division rather than cell elongation, which many BR mutants exhibit (Schaeffer *et al.*, 2001).

When compared with the *hydra* profiles, low levels of either sitosterol or campesterol lead to a reduction in growth and fertility. In particular, a reduction in sitosterol is linked with a reduction in cell division and plants that cannot be rescued by BR application. This is intriguing as the *smt1* mutants do not have reduced pools of campesterol, and there is even an increase in campesterol in the *SMT2* co-suppressed plants, and so this should not affect BR production as there should be no shortage of precursors. This therefore implies that for a BR response another sterol is required, possibly to perform another function other than acting as a precursor, and that the phenotypes of these mutants are not due solely to a deficiency in BRs.

Both *hydra1* and *hydra2* have reduced amounts of both campesterol and sitosterol, have reduced stature and are male sterile. Both also have a short root phenotype, however in *hydra2* cell division ceases within the root meristem, so if the amount of sitosterol was the key indicator of cell division activity then *hydra1* should have the same phenotype, as it has roughly the same level of sitosterol as *hydra2*. *hydra2* has no campesterol at all whilst *hydra1* has 12% wild type level, and so it could be that campesterol functionally replaces sitosterol in this role. However, *smt1* mutants also have low sitosterol, but normal levels of campesterol, yet they still exhibit reduced root growth and the cessation of division within the meristem of the worst roots (Diener *et al.*, 2000). High levels of campesterol are coupled with low levels of sitosterol in *SMT2* co-suppressed plants, and they have reduced cell division as well. So sitosterol may have a strong influence upon cell division.

There are some peculiarities with sterol biosynthesis that must be appreciated in order to fully understand these mutants and their sterol profiles. The predicted sterol pathway is not strictly linear. For example mutations in *SMT1* do not fail to interrupt the further transformation of cycloartenol into the normally dominant Δ5-sterols, sitosterol and campesterol (Diener *et al.*, 2000). It is therefore difficult to determine the normal substrates for these enzymes by analysing the accumulation of sterols within a mutant, as many of the biosynthetic enzymes may perform reactions in the mutants that they do not perform in the wild type (Diener *et al.*, 2000). For example *SMT1* and *SMT2* may functionally substitute for each other. Indeed, the action of these 'imposter' enzymes

can confuse the interpretation of the natural enzyme's roles. Interestingly the *hydra* mutants can still produce stigmasterol without accumulating sitosterol, it's supposed structural precursor. This may explain why the profile of the mutants is different from a plant that has been grown in the presence of chemical inhibitors of either of the two enzymes (Grandmougin *et al.*, 1989). Such chemicals may inhibit other enzymes within the pathway, whereas a genetic mutation is much more specific.

So far we have not discussed the influence of stigmasterol and yet both mutants accumulate stigmasterol at the expense of sitosterol, its structural precursor. This is due to a lack of experimental evidence, and the fact that no mutants prior to the *hydras* have implicated a role for stigmasterol in growth and development *in planta*. Sitosterol and campesterol are the major players in the plant cell, accounting for 19.51 and 4.2μg/g FW tissue respectively, while by comparison stigmasterol only accounts for 1.62μg/g FW tissue, and so in the wild type does not have a major presence. In the *hydra* mutants however, stigmasterol has replaced the two major sterols, sitosterol and campesterol, and become the major accumulated sterol. Insights into the role of stigmasterol come from studies using reconstituted membrane systems, where its influence upon the packing and ordering of the membrane was measured, and which we will look at next.

7.3 Sterols: Signal Precursors, Possible Sterol Signals, and Major Players in Membranes

Plants produce a complex mixture of sterols in comparison to both mammals and yeast. Mammals produce cholesterol as their major membrane sterol, whilst yeast produces ergosterol. Plants, however, tend to have a characteristic mixture of sterols, maize for example has a mixture of about 61 sterols (Hartmann, 1998).

7.3.1 Sterol-Lipid Interactions: Membrane Ordering is Dependent Upon Specific Sterol Species

The major products of the sterol pathway in plants are the membrane sterols campesterol, sitosterol and stigmasterol. Membrane sterols perform a bulk function, contributing to membrane fluidity, or the degree of motional freedom within the membrane, and permeability, as well as participating in the control of membrane-

associated metabolic processes. Sterols are incorporated into the plasma membrane after being transported from the endoplasmic reticulum, with a 3β-OH facing the water interface and the side chain extending into the hydrophobic core. Sterols therefore interact with the fatty acyl chains in phospholipids which in turn modulates the physical state of the bilayers through restricting the motion of the fatty acyl chains (Hartmann, 1998). This is known as the Sterol Ordering Effect, and it is instrumental in establishing lateral domains of ordered lipids within the membrane. Ordering is based upon the length and saturation of the lipid's acyl chains, and partitions them into domains of distinct fluidity and molecular ordering. Some regions of the membrane may be richer in some lipid species than others due to their mutual solubility properties. Cholesterol, however, actively reorganises the bilayer by preferentially associating with certain classes of lipid (Carruthers and Melchoir, 1986). In mammals lateral domains are thought to be involved in concentrating or compartmentalising reactions either on or in the membrane (Svetek *et al.*, 1999b), and have also been implicated in regulating cell adhesion and cellular signalling (Hoekstra and Van Ijzendoorn, 2000).

Lipid organisation is also affected by association with adjacent proteins or lipids, and, since sterols are also known to interact with the proteins within the membrane, a number of knock-on effects must be considered when looking at the influence of different sterols species within the membrane. In animal systems cholesterol has a role in lipid trafficking as well as sorting within the membrane (Hoekstra and Van Ijzendoorn, 2000), and so there may be an additional influence of the abnormal sterol profile in the *hydras* upon the state of the membrane. In support of such an influence, the *hydra* mutants also showed altered membrane lipid profiles (J. Topping and K. Lindsey, unpublished data, personal communication).

The accumulation of short chain fatty acids (SCFAs) in the plasma membrane causes an increase in fluidity, and has been linked with an increase in ethylene sensitivity (Whitehead and Vasiljevic, 1993). Though it is not known whether this is due to an increase in the availability of binding sites or to a direct interaction affecting the functioning of the receptor. Conversely, transgenic *Arabidopsis* seedlings, expressing a *35S::FATTY ACID ELONGATION1* (*FAE1*) construct, show gross changes in morphology that is linked to the accumulation of Very Long Chain Fatty Acids (VLCFAs) (Millar *et al.*, 1998). *FAE1* encodes a VLCFA condensation enzyme, the first in the biosynthetic pathway. In the transgenic *35S::FAE1* plants C20 and C22

chains accumulated at the expense of the wild type C16 and C18 chains. This was correlated with stunted growth, reduced elongation of the roots and shoot, reduced apical dominance and reduced fertility. The plants were altered in the shape and size of all of organs, suggesting that it had disrupted a process fundamental to development. Electron microscope analysis showed that the *35S::FAE1* plants possessed highly curved chloroplast membranes. Therefore the authors suggest that the altered shape of the cell membrane could indirectly influence the shape of the cell wall, possibly through an interaction with the cellulose synthase complexes that are present in the membrane. FA chain length can therefore dramatically alter the curvature of the membrane, as the hydrophobic tail of the VLCFA requires interdigitation with the opposite membrane leaflet. It is thought that normally this is required to stabilise highly curved membranes, such as around the nuclear pores in the nuclear envelope.

Although the *hydra* mutants do exhibit aberrant cell expansion, there is as yet no evidence of an alteration in the length of the FA chains, and no evidence of altered membrane curvature. Electron microscope analysis would be required to investigate this.

The types of sterol present in a membrane can, through interactions with the different lipid species, alter the topography of the membrane surface by altering its curvature, and consequently affect invagination and vesicle formation, as well as the recognition of external stimuli at the cell surface (Schuler *et al.*, 1991; Hoekstra and Van Ijzendoorn, 2000; Whitehead and Vasiljevic, 1993).

Some types of sterol are more able to order membranes whilst others are specifically required to control permeability of the membrane. Δ8-sterols, the precursors for the Δ5-sterols (sitosterol and campesterol), are far less efficient at ordering bilayers and altering the permeability of the membrane. Indeed, the Δ5-double bond is the most effective feature for optimal sterol-phospholipid interactions and regulation of membrane permeability (Ranadive and Lala, 1987). Sitosterol and campesterol are both able to reduce the permeability of the membrane, whilst stigmasterol, the other major Δ5-sterol, is not as efficient. Stigmasterol only differs from sitosterol by having a Δ22-double bond in its side chain, yet it has no ability to alter permeability (Schuler *et al.*, 1991). It

therefore appears that the Δ22-double bond is essential for the sterol to perform the two functions of acyl chain ordering and regulating membrane permeability.

Cell cultures have been grown in the presence of fungicides and herbicides which specifically inhibit sterol biosynthesis. Fenpropriomorph, N-substituted morpholines and azadecalines for example, which cause the normally abundant Δ5-sterols to be replaced by cyclopropylsterols (Grandmougin *et al.*, 1989). Cyclopropylsterols appear to be good surrogates for the Δ5-sterols, which is probably why cell lines can survive and eventually start to proliferate after a couple of generations. However, as mentioned above, these chemicals inhibit other enzymes within the pathway and thus create a different sterol profile to those found in the *hydra* mutants.

7.3.2 Sterol-Protein Interactions Within the Membrane

As mentioned, sterols also have an ability to modulate the activity of proteins and other enzymes within the membrane. The activity of H^+ATPases has been shown to be specifically modulated by sterols (Grandmougin-Ferjani *et al.*, 1997). Cholesterol and stigmasterol stimulated the proton pump, whilst sitosterol and campesterol reduced its activity considerably.

It is essential when trying to understand how the membrane environment could alter the activity of a membrane-bound protein, the possible interactions that may be taking place. If an enzyme crosses the bilayer then it is in contact with the bulk water region, the interfacial region, lipid head region, the lipid backbone region and the hydrocarbon core (Carruthers and Melchoir, 1986). Only a small fraction of the protein will be exposed to water, and so the interactions with the surrounding bilayer become the prominent feature in influencing the activity of the enzyme. It must also be remembered that some enzymes may only exist within specific lateral domains, determined by the presence of specific lipid species.

There are two theories to account for the ability of membrane sterols to modulate the activity of membrane-associated proteins. Firstly, changes in the sterols present in the membrane have a knock-on effect upon lipid composition, which alters the topography and fluidity of the membrane and causes a change in the protein's conformation, thus altering its ability to function (Cook and Burden, 1990). Alternatively, the annulus

hypothesis suggests a direct sterol-protein binding model (Simmonds *et al.*, 1982), in which membrane lipids bind strongly, and sterols weakly, to the annular sites, whilst being the opposite at the non-annular sites. Protein activity would therefore be determined by the interactions of the sterol molecules and the fatty acyl chains with both the annular and non-annular sites of the protein. It is most likely that both membrane fluidity and the specific interactions of the sterol and lipid species have an impact upon protein activity. Indeed, as mentioned above, there is good evidence that sterols can reorganise lipid packing in the bilayer and bring about domains of varying lipid composition. Enzyme activity is therefore closely correlated with both lipid and sterol composition, and thus any changes to the membrane environment will result in altered enzyme activity (Carruthers and Melchoir, 1986). However, if the absent sterol is functionally replaced by another, then this effect will be minimised.

It is important to note however that so far the only evidence for these interactions comes from reconstituted membrane systems, rather than direct *in planta* evidence.

7.3.3 Sterols as Signals in Morphogenesis: An Interesting START for the New Player

Sterols clearly play an essential role in the plasma membrane as well as being the precursors for the BR hormones (Hartmann, 1998), but do they have another role to play in morphogenesis? In trying to explain the phenotype of the *fackel* mutant, Schrick and colleagues postulated the existence of a novel, as yet unknown, sterol signalling molecule (Schrick *et al.*, 2001). This hypothesis has received support from the identification of sterol binding (START) domains in the proteins of a number of patterning genes (Ponting and Aravind, 1999). In particular, the homeodomain genes *PHABULOSA* (*PHB / ATHB14*), *PHAVOLUTA* (*PHV / ATHB9*), *GLABRA2* (*GL2 / ATHB10*), *REVOLUTA* (*REV*), and *ARABIDOPSIS THALIANA MERISTEM LAYER1* (*ATML1*) all contain START domains. However, at the moment it is unclear whether the START domain has any role in the function of these proteins, but the fact that the only alleles of *phb* and *phv* isolated so far have mutations within the START domain suggests that it is important for the normal functioning of these two proteins, and thus may be important in the others as well (McConnell *et al.*, 2001).

START domains were initially found in the protein kinase C family, in the first two subunits of the protein. Binding of a diacylglycerol (DAG) second messenger to the amino-terminal half of the protein allows catalysis to proceed by removing an autoinhibitory pseudosubstrate domain from the active site (Newton, 1995). The C1 subunit that binds the DAG molecule is homologous to a region of Steroidogenic Acute Regulatory Transfer Protein (START), which has been shown to bind sitosterol as efficiently as cholesterol, and which gives its name to the domain (Kallen *et al.*, 1998). START domains can also bind to membrane-derived phospholipid second messengers and to plasma membranes (Newton, 1995). So whether these proteins bind to the plasma membrane until activated, or a sterol ligand binds them in the cytoplasm and directs them to the nucleus is still to be seen, but GL2 has been shown to exhibit developmental stage-specific partitioning between the nucleus and the cytoplasm (DiChristina *et al.*, 1996).

The exact nature of the ligand and the ligand-START domain interaction for the homeodomain patterning genes is presently not understood, but the availability of alleles with point mutations in the START domain should allow further investigation of their role. It is likely there is a common ligand and mechanism of activation within some of these genes, for instance *in situ* localisation of the HD-ZIP genes *REV*, *PHB* and *PHV* show overlapping expression in the vascular tissues, the shoot meristem and the adaxial side of the emerging cotyledons in the developing embryo (Otsuga *et al.*, 2001; McConnell *et al.*, 2001). *REV* is important for regulation of the axillary meristems, for the regulation of cell division in the leaves, and for the correct development of the vasculature. Interestingly *CLV* and *STM* are both down regulated in *rev* mutants (Otsuga *et al.*, 2001). *PHB* and *PHV* are both required for adaxial cell fate in the aerial organs (McConnell *et al.*, 2001), a role for which *REV* may be implicated in as well. However the overlapping expression of these proteins may instead represent the common location of the sterol ligand, which is perhaps synthesised within the shoot meristem region, an area where many of the sterol biosynthetic genes are expressed (Souter *et al.*, 2002; Jang *et al.*, 2001; Schrick *et al.*, 2001; Diener *et al.*, 2000). In the model being proposed for the spatial expression of the PHB and PHV proteins, the sterol-based ligand (signal) is made within the shoot meristem, thus creating a signal gradient from the inside to the outside of the developing leaf primordia (McConnell *et al.*, 2001). The cells closest to the shoot meristem, on the adaxial side of the primordia, perceive the signal, activating the PHB and PHV proteins to enter the nucleus and

activate the transcription of the downstream genes required for the patterning of the adaxial side of the leaf. The differentiation of the adaxial side then induces the subsequent differentiation of the abaxial side, where the ligand signal cannot reach. Interestingly the *hydra* mutants show abnormal adaxial-abaxial cell fate specification, consistent with the view that the *HYDRA* genes are required for the production of the sterol ligand.

While there is certainly an interesting case for the theory that the absence of the sterol ligand creates the *hydra* mutant phenotype, it cannot wholly explain all of the defects seen in the mutants, a number of which can be rescued by inhibiting other signalling pathways, such as ethylene and auxin.

7.3.4 Mis-expression of *GL2::GFP* and the *hydra* Root Hair Phenotype

In the root, the mutants show defective root hair patterning and in particular mis-expression of the *GL2::GFP* construct in the *hydra2* background. *GL2* specifies the non-hair cell fate in the atrichoblasts of the root epidermal surface (Masucci *et al.*, 1996). Expression is regulated in a strict spatial pattern that relies on correct positional information as well as feedback inhibition between the neighbouring cell files (Lee and Schiefelbein, 2002). It is certainly interesting that *GL2::GFP* expression is seen in all cell files in *hydra2*, while hairs develop from all cell files. With the observed expression pattern there should be no root hairs at all.

There are two possible reasons for this. Firstly, the GL2 protein may not function properly in the *hydra* mutants because the START domain ligand is lacking. This could lead to the *hydra* root hair phenotype, however there is no evidence to corroborate the view that GL2 does function correctly. The second, and most likely reason, for the *hydra* root hair phenotype is that auxin and ethylene override the underlying patterning programme because they act downstream of *GL2* (Masucci and Schiefelbein, 1996). More wild type root hair patterning was seen in the *hydra axr1-12*, *hydra ein2* and *hydra2etr1-1* double mutants, and this suggests that defective auxin and ethylene signalling is responsible for the root hair phenotype. *axr3-1* mutants do not themselves develop root hairs (Leyser *et al.*, 1996; K. Knox and O. Leyser, personal communication), so the total lack of root hairs in the *hydra2axr3-1* double mutants does not support or discredit the idea that the GL2 protein is non-functional in the *hydras*.

However, this does not address the question of why *GL2* is mis-expressed in the first instance.

It could be that there exists a mechanism, as yet unknown, through which *GL2* expression is regulated by the GL2 protein through the START domain. However this is utter speculation as there is no experimental evidence to support this. None of the published *gl2* mutant alleles contain a mutation within the START domain of the protein, so the START domain may have no relevance to its normal functioning. Unfortunately the GFP construct used was a promoter-fusion rather than a protein-fusion, and so did not contain the START domain, which is only present in the protein. The expression pattern of a protein fusion may not show any difference in *hydra2* to that of the promoter fusion, however it would show any differences in the localisation of the protein within the cell. GL2 has been shown to exhibit developmental stage-specific partitioning between the nucleus and the cytoplasm (DiChristina *et al.*, 1996), however this has not been linked with the START domain.

A more probable and better supported hypothesis is that the positional expression of *GL2* is disrupted in *hydra2* because of defects in the cortical cell layers underlying the epidermis. The recent cloning of the *ECTOPIC ROOT HAIR3* (*ERH3*) gene adds some support to this hypothesis (Webb *et al.*, 2002). *ERH3* encodes a katanin-p60 protein which is believed to be involved in severing microtubules, and so may have a role in cell wall organisation during cell expansion and differentiation. Similar to the *hydra* mutants, *erh3-1* mutants develop root hairs in the non-hair position and mis-express *GL2*. The underlying cell walls of the cortex are not correctly aligned with the epidermal cell files, and so it has been proposed that the expression of *GL2* in these mutants is defective because of the spatial organisation of the signals that provide the positional information to the epidermis is disrupted (Webb *et al.*, 2002). If this is applied to the *hydra* mutants, it implies that the cortex in the *hydra* mutant root is similarly mis-aligned with respect to the overlying epidermal cell files. However, although no evidence has been presented in this thesis of an altered cortical cell layer, the abnormal shape of the epidermal cells seen in Figure 3.18 does support this hypothesis, as presumably only one cell layer need be mis-aligned for the positional signals to be affected. *GL2* expression within the epidermal layer is dependent upon the expression of *WER* and *CPC*, which feeds back and down-regulates the expression of *GL2* within hair cells to allow hair development to begin, whilst maintaining expression

in non-hair cells (Lee and Schiefelbein, 2002). If the mis-alignment of the epidermis has disrupted the positional signals from the cortical layers below, then it is also possible that the lateral feedback inhibition signalling mechanism is similarly impaired. The *erh3-1* mutant phenotype also implicates a possible role for microtubule arrangements in specifying cell identity, but we will return to this point later.

Clearly then, the complexity of the *hydra* phenotype cannot be explained by the disruption of a few patterning genes that may bind a sterol signal molecule that has not been identified yet. *GL2* is mis-expressed but makes no impact upon the phenotype because it is overridden by the defects in auxin and ethylene signalling acting further downstream. At present there are not enough data on the interaction between different sterols and the START domain of these proteins, and therefore we do not know how influential this could be on the phenotype of the *hydra* mutants. If the sterol signal is important, when is it important? Does it have a role in the spatial expression of these genes, and if so do they take their lead from other known spatial cues, such as auxin transport or auxin gradients?

As well as having a potentially important role in regulating the activity of proteins containing a START domain, the results presented in this thesis suggest that sterols have a more important role in integrating a number of key signalling pathways at the plasma membrane.

7.4 Sterols Integrate Signalling Pathways at the Plasma Membrane

Bulk sterols are major players in regulating the plasma membrane environment, creating the right conditions for the regulation of transport across the membrane by altering its fluidity and permeability, through ordering of the membrane lipids, as well as interacting with membrane-associated proteins. It is therefore possible to imagine the impact that replacing the ordering sterols (sitosterol and campesterol) with non-ordering sterols (stigmasterol) could have upon the membrane, and upon membrane-bound proteins that are involved in the perception or transport of hormones. In this section I will look at how such an alteration in bulk sterol production leads to the numerous signalling defects that we see in the *hydra* mutants. This evidence supports the hypothesis that the aberrant sterol profiles in the *hydra* mutants leads to abnormal interactions with membrane-bound proteins and to defects in membrane permeability.

7.4.1 Ethylene Signalling is Defective at the Plasma Membrane-Localised Receptor *ETR1*

The *hydras* do not detectably overproduce ethylene and are rescued by silver ions, which inhibit signalling at the receptor (Beyer, 1976; Rodriguez *et al.*, 1999). Mutations in *CTR1*, the immediate downstream component from the ethylene receptor, cannot be rescued by silver ions (Kieber *et al.*, 1993). Together these results suggest that the ethylene receptor is the source of the defective ethylene signalling in the *hydras*. They respond as if they are producing a large amount of ethylene, such that in *hydra2* it appears to be fully saturated. The addition of ACC does not increase the severity of the phenotype, whereas in *hydra1* the addition of ACC causes a further reduction in root growth. The introduction of a mutation in the ethylene receptor *ETR1* led to a more efficient rescue of root growth than with silver ions, consistent with the view that the ethylene receptor is defective in *hydra* ethylene signalling. In order to see how the ethylene receptor could be disrupted by the *hydra* mutations, we must first look in more detail at how the *ETR1* receptor functions and at how silver ions and the *etr1-1* mutation disrupts this.

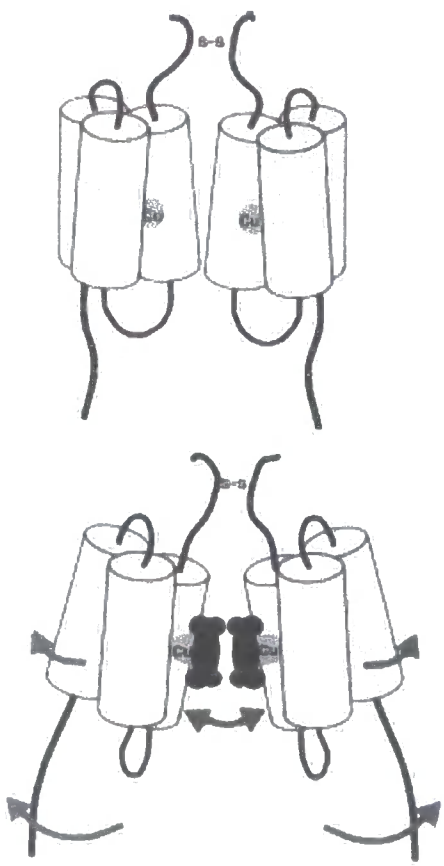
The membrane-localised *ETR1* protein has homology to the bacterial two-component receptors (Chang *et al.*, 1993), and can be divided into three domains of structural and functional significance; the N-terminal sensor domain, the C-terminus and the central core. The N-terminal region contains three membrane-spanning hydrophobic domains, possibly arranged as α-helices. Monomers are linked together as dimers through disulphide bridges at the N-terminus, which has an extra-cytoplasmic location (Chang *et al.*, 1993; Schaller *et al.*, 1995). The first 128 amino acids of the protein contains all of the elements necessary and sufficient for high affinity ethylene binding and thus constitute the sensor domain (Shaller and Bleeker 1995), which is intrinsic, within the membrane (Chang *et al.*, 1993; Schaller *et al.*, 1995).

Copper is the only metal ion that enhances ethylene binding, and a copper ion has been shown to be associated with the ethylene binding domain, in line with Burg's original hypothesis which predicted that this would be the case (Burg and Burg, 1967). The donation of h-electrons from ethylene to the d-orbitals of the transition metal result in a resonance structure that contains about half the binding energy of a carbon-carbon double bond (Muhs and Weiss, 1962). This provides the stability needed for high-affinity binding while allowing reversibility. It is believed that one copper ion is

embedded in the three membrane-spanning α-helices of each monomer. Residues 65 and 69 are essential for ethylene binding (Schaller and Bleeker, 1995; Schaller *et al.*, 1998), and modelling suggests that these two residues act to co-ordinate a copper ion in a hydrophobic pocket formed by the membrane-spanning helices. This pocket provides a favourable environment to stabilise a Cu (I) ion, which would allow ethylene binding (Thompson *et al.*, 1983).

According to the bacterial paradigm, the binding of the ethylene molecule with the copper complex either displaces or alters the co-ordination chemistry of the membrane-spanning α-helices and thereby induces a conformational change. This conformational change would be propagated by translational or rotational changes of the third transmembrane domain to the linked histidine kinase transmitter domains of each dimer pair (Bleeker *et al.*, 1998). This is represented in Figure 7.4.1 below.

Figure 7.4.1 Ethylene Binding Induces a Conformational Change in *ETR1*



Proposed mechanism by which binding of ethylene to a Cu(I) co-factor could induce a conformational change in the ethylene-binding domain of *ETR1*. Movement of the membrane-spanning α-helices would be propagated through the third helix to the histidine kinase transmitter domain. Mutations that disrupt ethylene binding (*etr1-1*, *etr1-3*, and *etr1-4*) could lock the receptor in one conformation. Mutations in the 3rd helix (e.g. *etr1-2*) could alter signal propagation without disrupting ethylene binding. (Taken from Bleeker *et al.*, 1998.)

The conformational change in the receptor allows signal output, which is only induced by the co-ordination of ethylene with the copper ion situated within the transmembrane

spanning domains of the receptor. Because the signal is propagated through the rotation of the third α-helix, it can therefore be envisaged that the state of the membrane influences this activity. Therefore, one possible explanation for the defect in ethylene signalling could be as a result of the interaction between the abnormal sterol and lipids profiles with the annular and non-annular sites in the membrane-spanning domains of the *ETR1* protein.

In *hydra2* stigmasterol, which is known to over-stimulate H^+ATPases activity (Grandmougin-Ferjani *et al.*, 1997), accumulates 322% more than in wild type, whilst the two sterols that are known to reduce the activity of the H^+ATPase, sitosterol and campesterol are 4% and 0% of wild type levels respectively. It is therefore possible that the alteration in the abundance of these sterols leads to a conformational change in the ETR1 protein, that resembles the change induced by ethylene binding. The inability of ACC to increase the severity of the phenotype could be due to the conformational change not allowing any further ethylene to bind. By contrast, *hydra1* does not exhibit a saturated ethylene response, and can be induced to phenocopy *hydra2* when supplied with ACC. It does not accumulate as much stigmasterol (182%) as *hydra2*, whilst it retains a small level of campesterol (12%). *hydra1* therefore exhibits a variable response, which could be due to domains of differential composition and fluidity within the membrane.

Before we discuss how the *etr1-1* mutation rescues the *hydra2* root phenotype, we must look at how silver ions inhibit ethylene signalling. In 1976 Beyer reported that silver was a potent inhibitor of ethylene action, but at the time the nature and mode of action of the ethylene receptor was unknown, and so a sceptical model of inhibition was proposed. At this time the presence of a copper co-factor had been predicted but had not been experimentally demonstrated, and so the action of the copper ion in co-ordinating the binding of ethylene and the propagation of the signal was not taken into account in the model. Therefore using evidence of known interactions between metal ions and ethylene, it was suggested that Ag (I) substitutes for Cu(I), thereby interfering with ethylene binding (Beyer, 1976). Outside the plant both Ag (I) and Cu(I) can form complexes with ethylene, however so can Hg(II), which is often used for trapping ethylene but which is a very bad inhibitor of ethylene action in plants. Therefore this initial model for the action of silver ions needed updating with respect to the evidence that has now accumulated on the ethylene receptor.

Lineweaver-Burk plot analysis shows that Ag (I) is a non-competitive inhibitor of ethylene action (Beyer, 1976), and in ethylene binding assays with the *Arabidopsis* ETR1 protein expressed in yeast showed that Ag(I) stimulated ethylene binding (Rodriguez *et al.*, 1999). This evidence suggests that silver inhibits ethylene action by altering the propagation of the signal that is initiated upon ethylene binding to the *ETR1* receptor. Indeed, Rodriguez and co-authors hypothesis that silver inhibits the conformational change that is required for signal propagation (Rodriguez *et al.*, 1999). In the *hydra* mutants silver ions could therefore alter the sterol-induced conformational change, as if ethylene is bound, and revert it back to a wild type inactive conformation, as if ethylene is not present. This could therefore explain the rescue of *hydra* root growth by silver ions. However, in order to test this further, competitive inhibitors of ethylene action, such as CO_2 or 1-MCP could be used and the extent of root growth recovery determined. CO_2 competes with ethylene for the binding site, whilst 1-MCP binds to the copper co-factor and inhibits ethylene binding for 4-7 days (Sisler and Serek, 1997).

Because ethylene is a negative regulator of the *ETR1* receptor family, mutations that disrupt ethylene binding or lock the receptor in one conformational signalling mode would be predicted to cause a dominant insensitive phenotype (Hall *et al.*, 1999). The *etr1-1* mutation represents one such mutation. In ethylene binding assays in yeast the *etr1-1* mutation completely abolished ethylene binding (Schaller and Bleeker, 1995), while biochemical studies have demonstrated that this is because the mutation abolishes the receptor's capacity to co-ordinate with the copper ion (Rodriguez *et al.*, 1999). It is therefore thought that the primary effect of the *etr1-1* mutation is to lock the receptor in one conformational signalling mode, and as a consequence be unable to co-ordinate with the copper ion and allow ethylene binding (Hall *et al.*, 1999). In the *hydra2* background the *etr1-1* mutation causes ethylene insensitivity, which would be expected because the *etr1-1* mutant receptor is locked in one conformation. Thus the *etr1-1* receptor appears to be insensitive to the influence of the abnormal sterol profile in the *hydra* membranes. This hypothesis satisfactorily explains how the ethylene defects are localised to the membrane-bound receptor, however *hydra2* would need to be crossed with other *etr1* alleles in order to confirm this model.

The *ETR1* receptor is therefore a membrane-bound protein that functions abnormally in the altered membranes of the *hydra* mutants, highlighting an important role for sterols in co-ordinating signalling pathways at the plasma membrane.

7.4.2 BRs Do Not Rescue the *hydra* Mutants: *BRI1*, Cell Division and Cell Expansion

This model of defective membrane structure and function predicts that membrane-bound hormone receptors other than *ETR1* could also be altered. As discussed earlier, BR biosynthetic mutants can be rescued with BRs (Szekeres *et al.*, 1996; Choe *et al.*, 1999a, b), apart from sterol biosynthetic mutants, such as the *hydras*, cannot. *smt1* mutants accumulate cholesterol at the expense of sitosterol whilst campesterol levels are normal, and are not rescued by BRs (Diener *et al.*, 2000). *35S::SMT2* transgenic tobacco plants accumulated sitosterol at the expense of campesterol, and were rescued by BRs, whilst co-suppression of *SMT2* resulted in low sitosterol but high campesterol levels; however the plants could not be rescued with BRs (Schaeffer *et al.*, 2001). The *hydras* lack sitosterol and, in *hydra2*, campesterol as well. A reduction in sitosterol can therefore be linked with an inability to respond to BR application. However, sitosterol is not a precursor of BR biosynthesis, and hence this raises an intriguing question. Why are mutants that lack sitosterol unable to respond to BRs?

BRI1 encodes a ubiquitously expressed plasma membrane localised receptor kinase, the only one specific to BRs present in *Arabidopsis*. It consists of a putative leucine-zipper motif, an extracellular domain containing 25 leucine-rich-repeats (LRRs) with a 70 amino acid island buried between the 21st and 22nd LRR, and a cytoplasmic kinase domain with serine/threonine kinase activity (Li and Chory, 1997). The *BRI1* extracellular domain has been shown to bind BR directly (He *et al.*, 2000).

If the correct functioning of the *ETR1* receptor is disrupted by the abnormal sterol and lipid profiles in the membranes, it is also possible that the correct functioning of *BRI1* could be similarly disrupted. Current models of *BRI1* signalling hypothesise that it is present either as a homodimer where BR binding could induce homodimerisation, or alternatively it exists as a heterodimer with another LRR-receptor kinase or an as yet unknown protein (Schumacher and Chory, 2000). The interaction with sitosterol could help to maintain the ability of *BRI1* to bind BRs by maintaining the 3-dimentional shape

of the extracellular LRR domain. Alternatively, sitosterol may facilitate homo-dimerisation either before or after BR binding, possibly through the ordering of specific lipid classes around the receptor itself, that may be essential for normal functioning of the receptor.

Interestingly BRs have been shown to induce expression of *cycD3*, even in the *bri1* mutant background, suggesting that activation occurs via a separate, *BRI1*-independent, mechanism (Hu *et al.*, 2000). However, *CYCAT:CDB:GUS* expression was not rescued in *hydra2* mutants grown in the presence of either 1μM or 10μM epibrassinolide. This would suggest that the *hydras* cannot perceive BRs through either mechanism. However it is important to note that defective ethylene signalling is a major factor in determining cell division within the *hydra2* root meristem, and so this may override the BR induction of *cycD3*.

Furthermore, BR signalling could be active but the end products of the pathway unable to perform their roles. BRs have a well documented role is cell expansion, which is why many BR mutants are called *dwarf* or *diminuto* (Choe *et al.*, 1999; Takahashi *et al.*, 1995), and thus it could be that the *hydra* mutants are unable to undergo correct BR-induced cell expansion. This could be because the delivery or activity of cell wall loosening enzymes or materials is inhibited through abnormal interactions of the vesicles and the membrane. However the correct delivery of PIN proteins to their cellular locations in both *hydra1* and *hydra2* suggests that vesicle delivery is not disrupted.

The *hydra* mutants have a short stature that may therefore be the consequence of not being able to produce or respond to BRs, but it must also be taken into account that BRs may not be able to override the effect of ethylene, which has a well documented role in promoting radial expansion at the expense of apical-basal expansion (Bergfeld *et al.*, 1988; Lang *et al.*, 1982; Roberts *et al.*, 1985). Inhibition of ethylene signalling in the *hydra2etr1-1* double mutants resulted in root cells that, when compared with *hydra2* mutants, had undergone greater expansion and had done so in a more uniform manner (Figure 5.4.2). Also, there are a number of membrane-bound elements that are required for normal cell expansion that may be disrupted due to the effect of the *hydra* mutations upon the state of the membrane. These include the interaction between the membrane-bound cellulose synthase complex and the cytoskeleton (Giddings and Staehelin, 1988,

Nicol and Hofte, 1998), or the budding of delivery vesicles (Hoekstra and van Ijendoorn, 2000). This may inhibit the induction of the expansion process itself. Indeed, the activity of H^+ATPases has been shown to be significantly modified by membrane sterols (Grandmougin-Ferjani *et al.*, 1997), and is involved in proton extrusion into the cell wall space, a process that is the first downstream response of auxin binding to *ABP1* (Barbier-Brygoo *et al.*, 1996).

The lack of sitosterol may be a key factor in modulating the activity of the *BRI1* receptor, but recent evidence suggests that it is directly involved in cellulose biosynthesis (Peng *et al.*, 2002). Cellulose microfibrils are made up of linked β-1,4-glucan molecules, which are added through the processes of chain initiation, elongation and termination. This occurs on the inner side of the cell membrane, with cellulose synthases forming a rosette complex that is integral within the membrane. The *CesA* genes are plant homologues of bacterial cellulose synthases, whilst the *KORRIGAN* (*KOR*) gene encodes a membrane associated cellulase that is also involved in secondary cell wall formation (His *et al.*, 2001). It is now apparent that CesA glucosyltransferase initiates glucan polymerisation by using sitosterol-β-glucoside as a primer (Peng *et al.*, 2002). The glucose is in a terminal linkage to sterol, more than 95% of which is specifically linked to sitosterol. Sitosterol-β-glucoside is synthesised by a UDP-glucose-sterol glucosyltransferase, and is also the primer for chain elongation. The *KOR* gene product may cleave the sterol from the growing glucan chain to allow further chain elongation, a possibility that is supported by the accumulation of lipid-linked cellodextrins in the *kor* mutant (Peng *et al.*, 2002; His *et al.*, 2001).

Considering that sitosterol has such an important role in this basic cellular process, it is possible that the lack of sitosterol in the *hydra* mutants affects cellulose biosynthesis. However a closer analysis of the cell walls in the mutants would have to be undertaken before its contribution to the phenotype could be assessed. The *hydras* do, however, offer a vehicle in which to further our understanding of sitosterol in cellulose microfibril deposition. They also facilitate the investigation of ethylene and membrane fluidity upon microfibril orientation. Membrane fluidity has been shown to influence microfibril orientation in the green alga *Boergesenia forbesii* (Mizuta *et al.*, 1994), and membrane shape has been suggested to influence cell wall deposition (Millar *et al.*, 1998).

7.4.3 Auxin Influx: Another Abnormal Sterol-Protein Interaction or a Defect in Membrane Permeability?

So far I have presented evidence for a defective interaction between the membrane-bound ethylene receptor *ETR1* and the abnormal sterol and lipid profiles in the *hydra* membranes, and postulated a similar interaction with the membrane-bound BR receptor *BRI1*. Sterols also have a well documented role in regulating permeability. Sitosterol and campesterol are able to reduce membrane permeability (Schuler *et al.*, 1991), and both are reduced or totally lacking in the *hydra* mutants. Stigmasterol is the major constituent of the *hydra* membranes, however it is not as efficient at regulating permeability (Schuler *et al.*, 1991).

When the *hydra* mutants are grown in the presence of 1μM NAA, an auxin analogue that is freely membrane permeable, they showed a hypersensitive callusing response. Similarly, concentrations of 2,4-D (0.1μM or 1μM) that did not induce a callusing response in wild type seedlings caused callusing in the *hydras*. 2,4-D is not readily membrane permeable, and requires the influx carrier for entry into the cell at high efficiency (Delbarre *et al.*, 1996; Marchant *et al.*, 1999). Consistent with the observed callusing response, the *DR5* reporter was up-regulated throughout the callus tissue, in the root and shoot regions. These results suggested that either there was an increased amount of auxin entering the cells, or that the cells were simply more responsive to the auxin that entered.

In order to investigate these possibilities mutant seedlings were grown in the presence of 10μM 1-NOA, a specific inhibitor of the influx carrier (Parry *et al.*, 2001), and 1μM 2,4-D. 1-NOA is a polycyclic derivative of acetic acid that is structurally similar to auxin but does not have any auxin activity (Parry *et al.*, 2001; Imhoff *et al.*, 2000). 2,4-D is a slow-diffusing auxin analogue that requires the influx carrier to enter the cell, and at a concentration of 2.2μM 1-NOA is able to inhibit influx of 1μM 2,4-D by 50% in tobacco protoplasts (Imhoff *et al.*, 2000). The presence of 1-NOA should therefore have made it more difficult for 2,4-D to elicit a callusing response as less of the carrier-dependent 2,4-D should have been able to enter the cells. However, under this treatment the mutants underwent a callusing response and the *DR5* reporter was up-regulated to the same degree as treatment with 2,4-D alone. When incubated with just 1-NOA, *DR5* expression was restricted to the root tip, and so the observed ectopic up-regulation of

DR5 throughout the seedling was due to 2,4-D entering the tissue. These pharmacological results suggest that the membranes in the *hydra* mutants may be more permeable than wild type to 2,4-D, as inhibition of the influx carrier still allowed up-regulation of the *DR5* reporter by the exogenous 2,4-D. However, this experiment does not exclude two alternative interpretations. It is also possible that the influx carrier has become 1-NOA-insensitive within the *hydra* background, or that the cells are more sensitive to the amount of 2,4-D that enters.

Therefore, to investigate further the interaction between the membranes in the *hydra* mutants and the influx carrier, a genetic approach was taken. Two different mutations in the *AUX1* influx carrier gene were introduced into the *hydra* backgrounds. The *aux1-7* mutation is a chemically-induced base pair substitution within the carboxyl-terminal portion of the protein, Gly^{459} to Asp^{459}, which alters its functionality and despite producing wild type levels of protein, it is effectively a null allele (Bennett *et al.*, 1996; A. Marchant, University of Nottingham, personal communication). The *aux1-100* allele is a T-DNA insertion mutant that creates a complete null allele; there is no *AUX1* protein detectable by Western blotting (A. Marchant, University of Nottingham, personal communication). The *aux1* mutants are characterised by a long agravitropic root, and the expression of the *AUX1* protein in the S2 layer of the columella has implicated it as a key mediator in the redistribution of auxin across the root tip early in the gravity response (Swarup *et al.*, 2001). Because this requires the specific functioning of the *AUX1* influx carrier, an indication of the effect upon auxin movement across the *hydra* membranes was achieved by subjecting the double mutants to a gravity response assay. However, before we discuss the implications of these results it is important to remind ourselves of the interactions of the two mutations in each of the *hydra* backgrounds.

Within the *hydra1* background both the *aux1-7* and *aux1-100* mutations caused an identical increase in root growth in the predicted double mutants. Both of the predicted *hydra1aux1* double mutants were gravity-responsive, representing a rescue of both the null (*aux1-100*) and the defective-protein (*aux1-7*) alleles. However the response kinetics of the two double mutants were different. The predicted *hydra1aux1-7* double mutants reoriented the growth of the root tip within the wild type response time. The predicted *hydra1aux1-100* double mutants reoriented towards the new gravity vector, but then grew beyond it before again reorienting.

hydra2 exhibited a different pattern of recovery. The predicted *aux1-7* heterozygous *hydra2* mutants showed an intermediate root growth increase when compared with the predicted double homozygous mutants. The predicted *hydra2aux1-100* double mutants, however, had a comparatively reduced increase in root growth, and did not show an intermediate phenotype within the predicted heterozygous *aux1-100 hydra2* seedlings. Both of the predicted double homozygous mutants responded to a change in the gravity vector. The predicted *hydra2aux1-7* double mutants responded slightly slower than wild type and grew directly towards the gravity vector. The predicted *hydra2aux1-100* double mutants were also slower in their response than wild type, but unlike the predicted *hydra1aux1-100* double mutants they grew directly towards the gravity vector.

The rescue of the gravity response of the *aux1-100* allele by both *hydra* mutations suggests that auxin is able to be transported across the root tip and distributed to elicit a differential growth response. The current model of auxin transport in mediating the gravity response hypothesises that the PIN3 protein becomes laterally distributed in the root meristem, predominantly to the upper side of the root tip, so as to transport more auxin towards the upper side of the root (Friml *et al.*, 2002b). PIN2 then transports auxin into the proximal and distal elongation zones, where on the upper side of the root cell expansion increases and brings about the turning of the root tip towards the gravity vector (Muller *et al.*, 1998; Rosen *et al.*, 1999). In *hydra1* and *hydra2* the delivery of these two PIN proteins was correct at the time the gravity response was carried out, and so the ability of the mutants to redirect auxin during the gravity response was not impaired. Therefore the limiting factor in the *hydra* mutants could be the amount of auxin available for relocation.

AUX1 protein is localised in the S2 layer of the columella, as well as in the protophloem of the root tip, and so has a role in providing the auxin for the mounting of the differential growth response (Swarup *et al.*, 2001). A cell that has an influx carrier will be able to transport auxin into it at a faster rate than a cell that does not have an influx carrier, as auxin will have to diffuse through the cell membrane. The *aux1-100* mutation causes a null allele, with no detectable protein made. Therefore in this mutant diffusion of auxin across the membrane of these gravity-important cells is the only way for it to enter the cell. However this means that there is an insufficient amount of auxin

entering the cells that express the PIN proteins that generate the differential distribution of auxin.

The rescue of the gravity response of the *aux1-100* mutant by the *hydra* mutants suggests that the membranes of both are more permeable to auxin than wild type. This would mean that auxin enters the cells in the meristem as a sufficient rate for the PIN proteins to mount a differential distribution of auxin across the root tip. It is also possible, however, that the membranes are not more permeable. Instead, the wild type level of auxin that diffuses through the membrane causes a response to be mounted because the cells are more sensitive to it.

Root growth is the combined product of cell division and cell expansion, and the different root growth rates of the two *hydra* mutants allows us to further interpret the *aux1-100* gravity response recovery. The predicted *hydra1aux1-100* double mutants re-oriented towards the gravity vector but kept growing past it and then re-aligning. In wild type seedlings the gravity response is mounted by the re-distribution of auxin across the root tip, a process that must be adjusted as the root realigns to the gravity vector, so that at the point when the tip points towards the vector the differential growth response has returned to a uniform one. This means that the gravity vector must be detected and the amount of auxin being differentially redistributed altered accordingly. Essentially then, the speed of the response machinery has to be fast enough so that the root does not grow past the gravity vector before uniform growth is restored. Since the growth rate of the two *hydra* mutants is different, with a slower growth rate seen in *hydra2* mutants, it means that the speed of response correlates differently to the growth rate in these two mutants. In *hydra1* the root tip realigns past the gravity vector and then initiates a recovery growth response to return to the vector. This suggests that the speed of growth outstripped the speed of auxin passing through the membrane, such that it responded to the initial reorientation of the vector, but as it re-orients it is unable to respond to the changes in the orientation of the root tip. In wild type roots the fine-tuning of the differential growth response ensures that the tip returns to a uniform growth response causing the tip to grow towards the gravity vector. It can therefore be suggested that the *hydra2aux1-100* double mutants grow towards the gravity vector because the speed of auxin redistribution is equal to the root growth rate.

In comparison the *aux1-7* double mutants showed a different interaction, one that supports the hypothesis that the abnormal sterol profiles of the *hydra* membranes results in abnormal interactions with membrane-bound proteins. The *aux1-7* mutation causes a carboxyl-terminal alteration change and produces a wild type level of protein (A. Marchant, Nottingham University, personal communication).

The predicted *hydra1aux1-7* double mutants responded within the wild type time frame, but in contrast to the predicted *hydra1aux1-100* double mutants, the roots grew directly towards the gravity vector and needed no readjustment. This suggest that the speed of auxin travelling across the membrane was equal to the speed of the readjustment response, which could be due to a slower rate of root growth in the *aux1-7* double mutants when compared with the *aux1-100* double mutant. However, when compared, the *hydra1aux1-7* and *hydra1aux1-100* double mutants showed similar rates of root growth (Figure 6.10.1). Therefore the aux1-7 protein was the reason for the difference in response, and it is possible that it had become functional again. A functional aux1-7 protein would be able to transport auxin into the cell at a faster rate than diffusion, such that the speed of auxin redistribution is equal to the root growth rate, ensuring that the root tip returns to uniform growth once it is pointing towards the gravity vector.

The reactivation of the *aux1-7* protein has a precedent. The application of 60μM chromosaponin I (CSI) was found to rescue the gravity response in *aux1-7*, but was unable to do so in the *aux1-22* allele, a T-DNA insertion null mutant similar to *aux1-100* (Rahman *et al.*, 2001). However, in wild type seedlings the application of CSI results in a loss of gravitropic growth. CSI is a γ-pyronyl-triterpenoid saponin isolated from pea, a sterol-derivatised saponin, that has detergent properties. Other detergents such Triton X-100, Tween 20, SDS and CHAPS do not disrupt the gravity response in wild type seedlings. In uptake assays, wild type CSI treated cells were unable to take up [^{3}H]IAA, whilst *aux1-7* CSI treated cells were able to take up the labelled auxin (Rahman *et al.*, 2001). These results show that CSI interacts with the *aux1-7* protein in a different manner to the wild type *AUX1* protein, reactivating it through a carboxyl-terminal interaction as is seen in the *hydra* mutant backgrounds.

In the *hydra2* background, the *aux1-7* allele exhibited a semi-dominant phenotype, which *aux1* mutants do not normally show (A. Marchant, Nottingham University, personal communication). However, if *aux1-7* is in the heterozygous state then it still

means that there is still one wild type *AUX1* protein being expressed, so why does it alter the phenotype?

Two wild type AUX1 proteins are present in the *hydra2* mutants, which have a short root phenotype. When one wild type AUX1 protein and one aux1-7 protein are present in *hydra2* there is an increase in root growth, and when both wild type AUX1 proteins are replaced with aux1-7 proteins, there is an even greater increase in root growth. If the aux1-7 protein was reactivated to the wild type level of activity, then there would be no difference in the phenotype of the AUX1/AUX1, AUX1/aux1-7 or aux1-7/aux1-7 protein combinations in the *hydra2* mutant. There are two possible reasons for this.

Firstly, the AUX1 protein might be functionally altered through abnormal sterol-protein interactions within the *hydra2* membrane, as ETR1 is. If this was the case then an inactive AUX1 protein would be equivalent to the *hydra2aux1-100* double mutant, and would also mean that the *hydra2* mutant was agravitropic. However the hydra2 and hydra2aux1-100 double mutants are not identical, when root growth rates of these mutants is compared there is a small increase in the *hydra2aux1-100* double mutants. Also the *hydra2exo* and *hydra2etr1-1* double mutants are not agravitropic, which is what would be expected if the AUX1 protein was inactivated. In support of this conclusion, the *aux1-100* mutation increases root growth in both *hydra1* and *hydra2*, a phenotype that would be expected only if the AUX1 protein was functional. *aux1* mutants do have longer roots than wild type, and this is thought to be due to the reduced amount of auxin that accumulates at the root tip (Swarup *et al.*, 2001). Localisation of the AUX1 protein to the protophloem poles situated behind the meristem, suggests that it has a role in facilitating the unloading of auxin from the phloem and supplying it to the root tip where it redistributed and enters the basipetal transport stream (Swarup *et al.*, 2001). Localisation of the protein in the phloem-loading regions of the shoot also suggests that it has a role in the export of auxin from the shoot, which presumably causes a feedback-inhibition of auxin biosynthesis (Kowalczyk and Sandberg, 2001), otherwise an abnormal shoot phenotype would result form the accumulation of active auxins in this tissue. The corollary of this is that the *aux1-100* mutation would reduce the amount of auxin accumulating at the tip of the *hydra2* root, which results in the elongation of the root through an increase in cell division.

Consequently, this suggests that the aux1-7 protein is not just reactivated through a carboxyl-terminus-membrane lipid-sterol interaction as in the *hydra1* background, it is hyperactivated. The result of the hyperactivation would be an increase in the flux of the auxin transport stream, or put another way, a reduction in the amount of auxin accumulating at the root tip. A reduction in the amount of auxin at the root tip would be equivalent to the effects of the *axr1-12* mutation, which caused an increase in root growth by reducing the responsiveness of the root tip to auxin. The *aux1-7* and *axr1-12* mutations therefore reduce the influence of auxin upon cell division activity within the meristem, resulting in a greater amount of root growth. This hypothesis is supported by the intermediate recovery of root growth by the *axr1-12* mutation in the heterozygous state.

In the *hydra1* background the *aux1-7* protein is reactivated, whilst in the *hydra2* background it appears to be hyperactivated. This interpretation implicates a key role for stigmasterol and/or campesterol in regulating the activity of the *aux1-7* protein. The presence of campesterol (12% of wild type) in *hydra1* may compensate for the accumulation of stigmasterol (182% of wild type), while the lack of campesterol in *hydra2* (0% of wild type) may be the reason for the difference in activation of *aux1-7*. However the level of stigmasterol may be the critical factor, as by comparison *hydra2* has 322% of wild type level.

Both the *aux1-100* and the *aux1-7* results support the hypothesis that the aberrant sterol profiles in the *hydra* mutants leads to abnormal interactions with membrane-bound proteins and possibly to defects in membrane permeability and/or sensitivity. The *aux1-100* results show that the membranes in the *hydra* mutant may be more permeable to auxins than wild type seedlings, but it is currently impossible to distinguish between the possibility of altered permeability and sensitivity to auxin. The *aux1-7* results provides further evidence of abnormal sterol-protein interactions. Although the impact of the interaction with the native AUX1 protein does not appear to be significant, the result shows that the defective sterol profile results in an abnormal interaction with a mutant version of the membrane-bound auxin influx carrier.

7.4.4 A Possible Interaction Between the *hydra* Membranes and the Cytoskeleton

The abnormal tip growth of the root hairs, which in wild type is driven by both auxin and ethylene (Pitts *et al.*, 1998), implicates an interaction with the cytoskeleton, which organises the delivery and deposition of the new cell wall (Giddings and Staehelin, 1988, Nicol and Hofte, 1998). The branched root hairs found in the *hydra* mutants can be reproduced by the chemical disruption of microtubules (Bibikova *et al.*, 1999). However, whether these are secondary effects of disruption in auxin and ethylene signalling or evidence of a direct interaction between components of the cytoskeleton and the abnormal sterol profiles in the membranes, that is masked by the hormone effects, remains to be seen. It may also be the case that the abnormal interaction is driven by the defective hormone signalling.

At the time of writing, F2 seedlings from a cross between the *hydra* mutants and an *ACTIN DEPOLYMERISATION FACTOR::GFP* (*ADF::GFP*) expressing line (courtesy of Patrick Hussey, University of Durham) are awaiting screening. Actin microfilaments are organised by *ADF*s, which bind to actin and regulate the polymerisation of microfilaments. *ADF::GFP* is expressed at the site of the bulge and also at the tip of the elongating root hair (Jiang *et al.*, 1997; Dong *et al.*, 2001), implying an early role in reorganisation to allow the polar localised outgrowth of the root hair. The main objective of this cross was to introduce a label of the actin cytoskeleton, so as to visualise it during development of the root hair, and thus be able to investigate the rescue of a normal expression pattern. Actin and myosin antibodies could also be used, however this is an invasive strategy and so GFP-labelled equivalents would be sought.

7.4.5 A Possible Interaction Between AGPs and Sterols

Another possible interaction of sterols, based on features of the *hydra* phenotype, is with the AGP family. *Arabidopsis* seedlings treated with the Yariv reagent, which specifically binds and cross-links AGPs, showed a reduced amount of growth of the shoot and root (Willats and Knox, 1996), and in roots this correlated with a reduced longitudinal cell expansion and increased radial expansion. The influence of such an interaction may also be compounded by ethylene, as inhibition of ethylene signalling in the hydra mutants increases rescues cell elongation, AGPs may still play a role in creating the *hydra* phenotype. Two mutants have been identified that have decreased AGP contents, *diminuto1* (*dim1*) (Takahashi *et al.*, 1995) and *root epidermal bulger*

(*reb1-1*) (Baskin *et al.*, 1992; Ding and Zhu, 1997). Interestingly *DIM1* is allelic to *DWF1* and *CBB1* and encodes a C-24 reductase, an enzyme that is late in the sterol biosynthetic pathway (Klahre *et al.*, 1998) (see Figure 7.2). *dim* mutants are BR mutants as they are rescued by the application of BRs. Although this may implicate an interaction between AGPs and BRs, the phenotype could simply be a secondary effect of BR deficiency. The *reb1-1* mutation can be mimicked by growing plants in the presence of Yariv reagent (Ding and Zhu, 1997). Bulging epidermal cells are common feature of the root epidermis in the *hydra2* mutants, and so adds further support to the possibility of abnormal AGP signalling.

AGPs are localised to the plasma membrane (Dolan and Roberts, 1995; Dolan *et al.*, 1995; Sherrier *et al.*, 1999), and *Arabidopsis* contains a family of 15 genes encoding the protein backbone of classical AGPs (Schultz *et al.*, 2000), many of which have GPI anchors and putative cleavage sites. GPI anchors help to anchor the protein to the cell surface in the same manner as a transmembrane domain, and can lead to increased lateral mobility in the membrane or to polarised transport to the apical surface of cells (Hooper, 1997). There are two ways in which the GPI-anchored AGP may function. In animal systems GPI-anchored proteins signal through interactions with other membrane-bound proteins, both in the same cell or in neighbouring cells (Peles *et al.*, 1997). An alternate mechanism of action is through cleavage of the protein from its lipid anchor by a phospholipase (Udenfriend and Kodukula, 1995, Oxley and Bacic, 1999), which could generate both intra- and extracellular proteoglycan components, some of which may be small enough (molecular mass ~20 kD; Fincher *et al.*, 1974) to move through cell wall pores (Carpita *et al.*, 1979).

The defective membranes in the *hydra* mutants could therefore influence the mobility of the GPI-anchored AGPs, or alter the ability of the phospholipase to cleave the protein. Alternatively the pores within the cell wall may be formed incorrectly, but this can only be determined by transmission and scanning electron microscopy or the cell wall. Either way, this is a potentially interesting area to investigate.

7.5 Conclusions

The *hydra* phenotype is a pleiotropy of features and influences. In this thesis I have focused mainly at the root, where the influence of defective auxin, ethylene and cytokinin has been investigated, resulting in the proposal of a model to account for the role of the *HYDRA* genes in creating these defects.

The evidence suggesting the ethylene defect is located at the receptor led to the proposal that abnormal sterol profiles in the *hydra* membranes interact with the *ETR1* receptor and result in its activation in the absence of ethylene. Support for this abnormal sterol-protein interaction comes from the reactivation of the *aux1-7* protein in both *hydra* backgrounds, and from the *ex planta* evidence discussed in the literature (Hartmann, 1998, Grandmougin-Ferjani *et al.*, 1997, Carruthers and Melchoir, 1986, Simmonds *et al.*, 1982, Cooke and Burdon, 1990). Therefore these findings provide the first *in planta* evidence for the influence of membrane sterols on membrane-bound proteins. In particular the loss of sitosterol and the accumulation of stigmasterol appears to be major factors in generating the abnormal interaction with the proteins.

The influence of sterols in regulating the permeability of the membrane is also well documented (Schuler *et al.*, 1991), however the *aux1-100* results provide the first indication of membrane sterols regulating the cell's permeability and possibly sensitivity to auxin. One consequence of the auxin-leaky membranes in the *hydra* mutants is that they would be unable to selectively compartmentalise auxin to specific cells and tissues. Stigmasterol is known to be a poor organiser of the membrane and be unable to regulate the permeability of the membrane (Schuler *et al.*, 1991), and in the *hydra* mutants, the accumulation of this disorganising sterol at the expense of the organising sitosterol and campesterol is consistent with the literature.

The overriding influence upon the *hydra2* root phenotype is ethylene, which causes a reduction in the amount of cell division and as a consequence the loss of patterning in the root tip. This may be due to the cessation of cell division as a result of the abnormal ethylene signalling (Herbert *et al.*, 2001), which could be compounded by the lack of sitosterol, a reduction in which has also been linked with a reduction in cell division (Diener *et al.*, 2000). Auxin also plays a role in creating the root phenotype, as reducing the responsiveness of the root results in an increase in root growth. However it has not been possible to determine if this is due to an accumulation of high levels of auxin at the

root tip or an increase in the sensitivity to auxin. The decrease in auxin at the root tip brought about by the *aux1-100* mutation (Swarup *et al.*, 2001), and possibly through the hyper-reactivation of *aux1-7*, supports the former, whilst the ability of low levels of the membrane-permeable auxin NAA to induce a callusing response supports the former. The contribution of abnormal cytokinin signalling to the *hydra2* phenotype early in development, between ca. days 3 and 6 post germination, is difficult to determine without the analysis of cytokinin mutations within the *hydra* background, and so nothing more can be concluded here.

The proposal that the *hydra* mutations lead to aberrant sterol profiles within the membranes allows for the interpretation of a number of the phenotypic characteristics of the mutants. Indeed, there are a number of possible interactions with different components of signalling mechanisms that either contain a membrane-localised protein or involve a direct interaction with the plasmamembrane itself. The patterning of root hairs is disrupted by defective ethylene signalling and possibly through the inability of the mutants to spatially localise the auxin signal in the epidermis due to leaky membranes. In the shoot the spatial pattern of stomatal development is disrupted, a process that involves the differentiating stomatal cell producing an inhibitory signal that stops the surrounding cells from following the same fate (Geisler *et al.*, 2000). Therefore the propagation, movement or perception of this signal is disrupted, as the *hydra* mutants develop large clusters of stomata, similar to the *TOO MANY MOUTHS* mutant (Geisler *et al.*, 2000; Yang *et al.*, 1995). Vascular patterning is also disrupted in the mutants, with isolated vascular elements separated from the disorganised main vasculature. Auxin is well documented in its role in inducing vascular differentiation through the precise transport of auxin, or canalisation of the auxin stream (Sachs, 1981, Aloni and Zimmerman, 1983, Aloni, 1987, Mattsson *et al.*, 1999, Galweiler *et al.*, 1998). So the inability of the *hydra*s to spatially restrict this signal may be the cause of this phenotype. However the severity of the vascular defects are no doubt highly influenced by the defective cell division and expansion that occurs in the aerial organs and in the hypocotyl.

The evidence presented in this thesis demonstrates that bulk membrane sterols perform an essential function in integrating and regulating auxin, ethylene and cytokinin signalling pathways at the cell membrane, and thus shows the role of the *HYDRA* genes in the regulation of plant growth and development.

7.6 Future Work

A number of further experiments that would be beneficial in testing the hypothesis presented in this chapter have been mentioned already. However there are a number of other areas in which future work could be carried out that have not been discussed thus far, and that would lead to a greater understanding of the role of the *HYDRA* genes and sterols in growth and development.

7.6.1 Transactivation of the *HYDRA* Genes Within Specific Regions of the *hydra* Embryo

Embryogenesis in the *hydra* mutants is disrupted form the globular stage onwards, however the precise manipulation of *Arabidopsis* embryos is very difficult, especially the exogenous delivery of substances. This makes any attempts to rescue development of the embryo particularly difficult by conventional methods, and so requires a molecular genetic approach. It would be particularly interesting to investigate the influence of the bulk membrane sterols in embryogenesis and apical-basal patterning.

The *hydra* mutants provide ideal subjects for this study, and the transient expression of either of the *HYDRA* genes would result in a rescue of the wild type sterol levels within the embryo. The *HYDRA* genes could even be discreetly expressed within specific regions of a *hydra* embryo using the *mGAL4-VP16* - *UAS* transactivation system (Haseloff, http://www.plantsci. cam.ac.uk/Haseloff/). The bacterial *mGAL4-VP16* transactivator can be fused to a plant specific promoter, such as *STM*, *DR5* or *LTP1*, and then transformed into a plant. A second construct is then made, fusing the *UAS* promoter, which is specifically activated by the *mGAL4-VP16* protein, to either of the *HYDRA* genes, and which would then be transformed into the corresponding heterozygous *HYDRA* line. It would be important to visualise the expression of this construct, and so either the GUS reporter gene could be added, or even better, a *HYDRA-GFP* protein fusion, provided that it did not alter the functioning of the biosynthetic enzyme. Once a *hydra* heterozygous line was obtained that was homozygous for the second construct, then the different combinations of specific promoter-activated *mGAL4-VP16* lines could be introduced into the different *hydra* backgrounds by crossing. The first generation of embryos would be screened for alterations in the patterning, with the expression of the GFP/GUS reporter being used to identify the correct embryos within the segregating populations.

Through the different combinations of transactivators, the importance of the *HYDRA* genes in each region of the embryo, and even within specific clonal groups of cells, can be determined. The transgenic lines could also be allowed to germinate so that the impact of embryonic recovery can be determined in post-embryonic growth.

7.6.2 Biophysical Techniques to Determine the Permeability and Fluidity of the Mutant Membranes

In order to build upon the model of altered permeability and fluidity within the membranes of the *hydra* mutants, it is essential to gain some data on the physical properties of the membranes. Two ways in which this could be achieved are fluorescence polarisation and electron paramagnetic resonance spectroscopy.

Membrane fluidity can be measured using fluorescence polarisation analysis of membranes labelled with fluorescent fatty acid analogues as probes (Kluge *et al.*, 1999; Bohn *et al.*, 2001). Two commonly used probes are the lipophilic DPH and TMA-DPH. DPH is incorporated into the hydrophobic (inner) part of the membrane, while TMA-DPH interacts with the hydrophilic (outer) part of the bilayer (Bohn *et al.*, 2001). The mobility of the probe incorporated into the hydrophobic domains of a membrane is strongly restricted if the fluidity of the membrane is low. This leads to correspondingly high values of fluorescence polarisation. If the membrane becomes more fluid then the motility of the probe increases and thus the values of fluorescence polarisation decrease (Kluge *et al.*, 1999).

The only limitations upon this technique are that it is performed upon reconstituted membrane fractions that are isolated from whole plants, and although the state of the art technology for isolating membranes, phase partitioning, could be used, it could lead to artefacts. Indeed the processes of protoplast preparation and membrane isolation are thought to alter the lipid composition of the membrane, and so it is therefore an advantage if membrane fluidity can be measured *in situ* (Svetek *et al.*, 1999b).

Electron paramagnetic resonance (EPR) spectroscopy provides such a method. EPR also requires the incorporation of a probe that can then be followed, except instead of fluorescence, EPR uses a free paramagnetic radical group (usually a nitroxide) which forms part of the spin-label. By realigning the spin-labelled probe and then releasing it

again, the subsequent movement of the probe within the membrane can be followed (Svetek *et al.*, 1999b). For example, the spin-labelled derivative of the methyl ester of palmitic acid, MeFASL, is readily distributed throughout the membrane due to its high hydrophobicity, and reports from the region close to the polar heads of the lipid molecules in the bilayer (Schara *et al.*, 1990).

Using this technique, Milan Schara's group in Slovenia have devised a protocol for measuring membrane fluidity *in situ* (Svetek *et al.*, 1999b). The roots of seedlings grown on the surface of agar can be used, they are removed form the plant, washed with the probe, blotted dry and then sucked into a glass capillary tube that is sealed at both ends. The capillary tube is then loaded into the EPR spectrometer, and measurements taken. This technique is more appropriate for the *hydra* mutants as the isolation of enough membrane material from the small *hydra* mutants could be a problem, as well as generating artefacts. The *in situ* nature of this approach is therefore crucial.

An interesting relationship to investigate would be the influence of ethylene upon membrane fluidity. There is evidence that during senescence the cell membrane becomes more fluidity through an increase in the sterol to phospholipid ratio, a process that is promoted by ethylene. DPH-labelled membranes showed an increase in microviscosity that was inhibited by AVG and silver ions (Thompson *et al.*, 1982). It would therefore be interesting to test *hydra2etr1-1* double mutants to see if there is any difference in the fluidity of the membranes when compared with *hydra* mutants.

7.6.3 Further Investigation of the AUX1 – Sterol Interaction

At the moment there are no data on the exact nature of the interaction between CSI and the aux1-7 protein, but the carboxyl-terminus is implicated as this is the site of the mutation. There are two possible explanations for the effect of CSI. Either the aux1-7 protein is reactivated in the membrane through an abnormal interaction between the detergent directly or indirectly through its influence upon the local composition of the membrane; or the *aux1-7* mutation causes the protein to be misdirected within the cell, as the AUX1 protein has been shown to cycle, like the PIN proteins (Geldner *et al.*, 2001), in a BFA-sensitive manner (M. Bennett, Nottingham University, personal communication). CSI could therefore interact either directly with the protein and lift the inhibition of its correct cycling and cause its installation within the membrane, or it

could interact with the cycling machinery directly. However, this is difficult without a specific AUX1 antibody. AUX1 was visualised by use of an epitope-tag, a nine amino acid hemagglutinin (HA) motif was fused to either terminus of AUX1 under the control of the native promoter and expressed in the *aux1-22* null allele background (Swarup *et al.*, 2001). It would therefore require the construction of an HA-tagged aux1-7 recombinant protein, with only the amino terminus epitope-tagged, as a carboxyl terminal tag could interfere with the interaction with CSI. The *hydra* results presently support an interaction between the membranes and the protein.

Uptake of [^{3}H]IAA by the roots of the *hydra aux1-7* and *hydra aux1-100* double mutants could be compared directly with *hydra* mutants, to gain some quantitative data on the different uptake capabilities of the three mutants.

7.6.4 Ongoing Projects

There are a number of ongoing experiments that have not been included in this thesis. Double mutants have been made with *pin3* and *pin4*, and are currently awaiting analysis. *pin3* is another agravitropic mutant and so the rescue of this would be very interesting but is not expected (Friml *et al.*, 2002b), although the *pin2/eir1* mutant is not rescued in the *hydra2* background. The most interesting aspect of this cross is the fact that *PIN3* expression is abnormal in the *hydra2* mutants, and so it could have an effect upon the loss of the columella root cap, facilitating a further understanding of the series of events that lead to this. The *pin4* mutant cross is also interesting as *PIN4* is expressed in the promeristem itself, taking auxin unloaded from the phloem and transporting it to the meristem and the distal root cap, creating a source of auxin for the basipetal transport stream (Friml *et al.*, 2002a). *pin4* mutants are unable to establish the tip-high gradient of auxin and so the reduction in auxin reaching the promeristem in the *pin4* double mutants would be expected to cause an increase in the amount of root growth. Therefore the cross would provide more evidence on the spatial influence of auxin upon the *hydra2* root meristem.

SCR is required for the correct specification of the endodermis in both the root and hypocotyl (Di Laurenzo *et al.*, 1996; Wysocka-Diller *et al.*, 2000), so in view of the defective patterning of the hypocotyl in the *hydras* a *SCR::GFP* marker line (courtesy of P. Benfey), was crossed into both mutant backgrounds. The F2 population is

currently awaiting analysis. The aim of this cross was to have a visual marker of cell identity within the radially expanded and disorganised hypocotyl, in effect an initial experiment into the further analysis of the hypocotyl.

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Appendices

Appendix 1 Mutants, GUS and GFP Lines Used

Mutant	Allele	Source
auxin resistant1	*axr1-12*	Ottoline Leyser
auxin resistant1	*aux1-7*	Malcolm Bennett
auxin resistant1	*aux1-100*	Alan Marchant
auxin resistant3	*axr3-1*	Ottoline Leyser
ethylene insensitive root1 (pin2)	*eir1-1*	Christian Luschnig
ethylene insensitive2	*ein2-1*	Stock Centre
ethylene resistant1	*etr1-1*	Stock Centre
pin-formed3	*pin3*	Jiri Friml
pin-formed4	*pin4*	Jiri Friml
GUS Marker Line	**Sign**	**Source**
ACC SYNTHASE1	*ACS1*	Marc van der Staeten
CYCLIN B CYCAT DB	*CYCAT*	Marie-Therese Hauser
DR5	*DR5*	Jane Murfett
EXORDIUM	*exo/EM101*	Keith Lindsey
IAA2	*IAA2*	Alan Marchant
LIPID TRANSFER PROTEIN1	*LTP1*	Sacco De Vries
PIN-FORMED1	*PIN1*	Klaus Palme
VASCULAR TISSUE AND TAPETUM1	*VT1*	Keith Lindsey
GFP Marker Line	**Sign**	**Source**
ACTIN DEPOLYMERISATION FACTOR	*ADF*	Patrick Hussey
GLABRA2	*GL2*	John Schiefelbein
SCARECROW	*SCR*	Philip Benfey

Appendix 2 PCR Conditions

RT-PCR Reaction Cocktail

AMV/Tfl 5x Reaction Buffer	10µl
10mM dNTPs	1µl
25mM $MgSO_4$	3µl
AMV Reverse Transcriptase (5u/µl)	1µl
Tfl DNA Polymerase (5u/µl)	1µl
Specific Forward Primer	50pmol
Specific Reverse Primer	50pmol
RNA	X
Nuclease-Free Water (to a final total volume of 50µl)	

PCR Reaction Cocktail

10X NH_4 PCR buffer	2µl
10mM dNTPs	0.4µl
50mM $MgCl_2$	0.8µl
Taq Polymerase	0.4µl
DNA Template	1µl
Specific Nested Forward Primer (10pmol stock)	2µl
Specific Nested Reverse Primer (10pmol stock)	2µl
sdH_2O (up to 20µl)	11.4µl

Appendix 2 PCR Conditions

RT-PCR Program

First Strand cDNA Synthesis

Reverse transcription	48°C	45 min
AMV RT inactivation and RNA/cDNA/primer denaturation	94°C	2 min

Second Strand cDNA Synthesis and PCR Amplification

Segment 1	94°C	30 secs
Segment 2	60°C	1 min
Segment 3	68°C	2 min
Cycle Count 40		
Final extension	68°C	7 min

PCR Program

Denaturation	94°C	30 secs
Annealing	55-60°C	1 min
Extension	68°C	1 min 30 secs
Cycle Count 40		
Final Extension	68°C	7 mins

Appendix 3 Root Growth Data

hydra1 Ethylene				
DAG	*hydra1*	AVG	$AgNO_3$	ACC
6	1.43 ± 0.15	2.33 ± 0.15	6.5 ± 7.1	1.1 ± 0.1
9	6.04 ± 0.24	4.8 ± 0.63	14.2 ± 2.09	4.6 ± 0.16
12	12.46 ± 0.72	6.0 ± 0.7	19.8 ± 2.22	6.5 ± 0.73
15	16.39 ± 0.76	6.35 ± 0.5	23.3 ± 2.34	

hydra2 Ethylene				
DAG	*hydra2*	AVG	$AgNO_3$	ACC
6	2.07 ± 0.16	1.9 ± 0.28	4.3 ± 0.26	1.75 ± 0.13
9	3.18 ± 0.19	2.6 ± 0.16	5.0 ± 0.26	2.8 ± 0.08
12	3.68 ± 0.21	3.1 ± 0.23	5.6 ± 0.37	3.2 ± 0.17
15	4.14 ± 0.23	3.44 ± 0.17	6.4 ± 0.4	

hydra2exo						
DAG	*hyd2EXO/EXO*	+ $AgNO_3$	*hyd2EXO/exo*	+ $AgNO_3$	*hyd2exo/exo*	+ $AgNO_3$
6	2.07 ± 0.22	2.07 ± 0.15	2.25 ± 0.35	2.25 ± 0.35	2.5 ± 0.16	2.5 ± 0.16
9	3.18 ± 0.49	4.3 ± 0.26	4.0 ± 0.47	5.2 ± 0.08	5.5 ± 0.19	6.93 ± 0.17
12	3.68 ± 0.56	5.0 ± 0.26	6.6 ± 0.46	6.9 ± 0.2	8.6 ± 0.21	11.0 ± 0.42
15	4.14 ± 0.62	5.6 ± 0.37	7.25 ± 0.61	8.34 ± 0.3	10 ± 0.23	13.56 ± 0.62
18	4.25 ± 0.69	6.4 ± 0.4	8.1 ± 0.59	9.78 ± 0.42	12.1 ± 0.19	16.12 ± 0.74
21	4.29 ± 0.46	7.01 ± 0.41	8.4 ± 0.6	11.0 ± 0.42	12.9 ± 0.18	18.05 ± 1.23

hydra2etr1-1		
DAG	*hydra2*	*hydra2etr1-1/etr1-1*
6	2.07 ± 0.16	4.69 ± 0.25
9	3.18 ± 0.19	7.39 ± 0.46
12	3.68 ± 0.21	12.19 ± 0.96
15	4.14 ± 0.23	22.39 ± 1.63
18	4.25 ± 0.19	34.58 ± 2.21
21	4.29 ± 0.18	44.05 ± 2.64
24		54.73 ± 3.59

Appendix 3 Root Growth Data

hydra1ein2		
DAG	***hydra1***	***hydra1ein2/ein2***
6	1.43 ± 0.15	5.54 ± 0.33
9	6.04 ± 0.24	9.4 ± 0.34
12	12.46 ± 0.72	16.27 ± 0.64
15	16.39 ± 0.76	20.0 ± 0.82
18	21.0 ± 0.89	28.11 ± 1.07

hydra2ein2			
DAG	***hydra2EIN2/EIN2***	***hydra2EIN2/ein2***	***hydra2ein2/ein2***
6	2.07 ± 0.16	3.29 ± 0.15	4.9 ± 0.19
9	3.18 ± 0.19	4.33 ± 0.22	6.1 ± 0.25
12	3.68 ± 0.21	4.78 ± 0.23	8.57 ± 0.67
15	4.14 ± 0.23	5.33 ± 0.24	9.43 ± 0.84
18	4.25 ± 0.19	6.0 ± 0.31	10.5 ± 0.88

hydra1axr1-12		
DAG	***hydra1***	***hydra1axr1-12/axr1-12***
6	1.43 ± 0.15	2.2 ± 0.1
9	6.04 ± 0.24	6.79 ± 0.26
12	12.46 ± 0.72	16.71 ± 1.08
15	16.39 ± 0.76	20.86 ± 1.49
18	21.0 ± 0.89	25.71 ± 1.97
21	26.79 ± 1.05	35.36 ± 3.25

hydra2axr1-12			
DAG	***hydra2AXR1/AXR1***	***hydra2AXR1/axr1***	***hydra2axr1/axr1***
6	2.07 ± 0.16	2.7 ± 0.05	4.2 ± 0.22
9	3.18 ± 0.19	3.83 ± 0.13	5.75 ± 0.22
12	3.68 ± 0.21	4.85 ± 0.13	7.16 ± 0.23
15	4.14 ± 0.23	5.38 ± 0.15	8.04 ± 0.26
18	4.25 ± 0.19	5.75 ± 0.15	9.04 ± 0.31
21	4.29 ± 0.18	7.04 ± 0.32	10.3 ± 0.44

Appendix 3 Root Growth Data

hydra1aux1			
DAG	***hydra1***	***hydra1aux1-7***	***hydra1aux1-100***
6	1.43 ± 0.15	3.0 ± 0.22	2.71 ± 0.17
9	6.04 ± 0.24	8.4 ± 0.49	8.75 ± 0.82
12	12.46 ± 0.72	16.9 ± 0.96	15.25 ± 1.17
15	16.39 ± 0.76	21.95 ± 1.35	22.2 ± 1.52
18	21.0 ± 0.89	25.5 ± 1.47	26.7 ± 1.4

hydra2 aux1				
DAG	***hyd2***	***hyd2aux1-100***	***hyd2AUX1-7/aux1-7***	***hyd2aux1-7/aux1-7***
6	2.07 ± 0.16	3.13 ± 0.08	2.29 ± 0.1	3.38 ± 0.24
9	3.18 ± 0.19	4.5 ± 0.14	3.63 ± 0.2	4.63 ± 0.31
12	3.68 ± 0.21	5.04 ± 0.16	4.64 ± 0.24	6.13 ± 0.31
15	4.14 ± 0.23	5.42 ± 0.18	5.0 ± 0.22	7.0 ± 0.0
18	4.25 ± 0.19	5.88 ± 0.2	5.57 ± 0.25	7.75 ± 0.14
21	4.29 ± 0.18	5.96 ± 0.22	6.07 ± 0.25	8.63 ± 0.38

hydra2eir1			
DAG	***hydra2***	***hydra2EIR1/eir1***	***hydra2eir1/eir1***
6	2.07 ± 0.16	3.09 ± 0.14	1.9 ± 0.05
9	3.18 ± 0.19	4.46 ± 0.19	2.7 ± 0.1
12	3.68 ± 0.21	5.2 ± 0.31	2.83 ± 0.11
15	4.14 ± 0.23	5.65 ± 0.34	3.03 ± 0.11
18	4.25 ± 0.19	6.28 ± 0.41	3.1 ± 0.1